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It is characteristic of Science and Progress that they continually open new fields to our visions.—PASTEUR.

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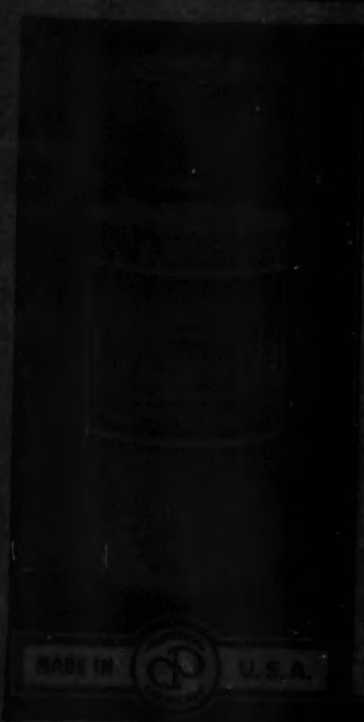

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A STUDY OF THE FORMATION OF GUM LEVAN FROM SUCROSE

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A large number of species of bacteria are known to produce gums from sugars. These may be classified into two main classes according to whether they form capsules, like the *Leuconostoc mesenteroides*, and *Semiclostridium commune*, or whether such capsules are absent, as in the case of Kramer's (1889) *Bacillus viscosus-sacchari*, Fritz Glaser's (1895) *Bacterium gelatinosum betae*, or Ritsert's (1891) *Bacterium gummosum*. These gum-forming species of bacteria may be still further classified according to the type of sugars which they are capable of utilizing for gum production. There are those on the one hand which form gum from either sucrose or invert sugar, others only from sucrose; one has been described which forms it from sucrose or maltose, and another from sucrose or raffinose. The species of bacteria comprising these three classes are as follows:

I. Gum formed either sucrose or invert sugar
   Leuconostoc mesenteroides
   Bacterium gelatinosum betae (Glaser)
II. Gum formed only from sucrose
   Bacterium gummosum (Ritsert, 1891)
   Bacillus gummosum (Happ, 1893)
   Bacillus levaniformans (Smith, 1901, 1902a)
   The Ginger Beer plant of Ward's consisting of *Bacterium vermiforme* and *Saccharomyces pyriformis*. (1899)
III. Gum formed from sucrose or maltose
   Micrococcus gummosus (Happ, 1893)
IV. Gum formed only from sucrose or raffinose
   Bacterium eucalypti (Smith, 1902b)

The species of bacteria of the first group are generally supposed to secrete invertase, and hence the gum that they form

is thought to be from the product of the inversion of the sucrose rather than from the sucrose itself. It is quite different with the second class, in which with the exception of the Ginger beer plant, no action of invertase is indicated. Its presence is inferred in the latter case only because of the fact that a true saccharomycete is involved in the transformation of the sucrose into gum.

In studying the gum-forming ability of the species of bacteria which he isolated from raw cane sugars, and which he named *Bacillus levaniformans*, Greig Smith (1901, 1902 a) discovered that it produced this gum only from sucrose. Neither glucose nor levulose, nor any combination of these sugars, was suitable as material for the formation of gum by these organisms. He concluded therefore that gum levan could only be formed from glucose or levulose in their nascent condition, thus assuming the action of invertase, where its presence had never been proved. The proof or disproof of this theory seemed relatively simple to the writer, who several years ago carried out some experiments in which levan formation in the presence and absence of growing yeasts was compared (1911 a and b). The results of these experiments showed that the amount of gum levan formed from the mixed inoculation of yeast and bacteria was never more than one-half, and frequently only one-tenth of that formed by pure cultures of the bacteria. These results showed that the relationship between yeast and bacteria was not a symbiotic one, and suggested that the presence of invertase deprived the bacteria of a suitable form of sugar for gum production. The conditions of those experiments were not entirely free from criticism, and the conclusions drawn from them might be objected to on the possible grounds that the relationship between the yeast and the bacteria were competitive. The decreased production of levan might be regarded as due to the inhibiting effect of the yeast upon the development of the bacteria rather than to the transformation by the former of the sucrose into sugars that the bacteria could not utilize. In order to remove such objections it was decided to use invertase instead of inoculation with yeasts. The amount of invertase added was to be so regulated that the

bacteria would have a slowly diminishing supply of sucrose to act upon, or if, as has been claimed, nascent glucose or levulose is the source of the gum, the microorganisms would be provided with a steady supply of this material in quantities commensurate with their power of assimilation.

PLAN OF THE EXPERIMENTS

The culture solution used for testing the gum-forming power of the bacteria was that used by Greig Smith (1902 a). The formula was modified as regards its sucrose content, as we used a 20 per cent instead of a 10 per cent sucrose solution. This was necessary in order that the period of inversion might be sufficiently prolonged so that it might proceed throughout the entire incubation period. The formula was as follows:

	per cent
Sucrose	20
KCl	0.5
Na_2HPO_4	0.2
Pepton	0.1

Two hundred cubic centimeter portions of this solution were poured into 300 cc. Erlenmeyer flasks, which were plugged with cotton and sterilized for thirty minutes on each of three successive days. The contents of the flasks were then inoculated with a pure culture of the levan-forming bacteria that had been isolated from a raw sugar. The inoculations were always made from forty-eight-hour cultures of the bacteria in the above solution. The invertase used in the experiments had been prepared by Kopeloff (1920) from Fleischmann's yeast. The invertase was added to the flasks with a sterile pipette, and they were then incubated for a period of ten days at 32°C. The analytical determinations were made as follows: The densities were determined by the Abbé refractometer, and expressed in degrees Brix. Sucrose was determined by the polariscope, the Clerget method of double polarization being used, and reducing sugars determined by the volumetric method of copper reduction, using Soxhlet's solution and Ross's (1912) method of testing for unreduced copper. For the determination of gum levan the

method of Kopeloff and Taggart (1920) was used. This method consists in calculating the levan present from the difference between the Clerget values by the acid and invertase method of inversion. The factor of 1.27 is used to convert this difference in values into the per cent of gum. This factor is derived from the change in specific rotation of gum levan from −40 to −92.5 when converted into fructose by hydrolysis with the acid used for inversion. This difference of −52.5 between the Clerget and the invertase values in the specific rotation of gum corresponds to 0.787 of the specific rotation of sucrose, which is 66.5. Hence 1.27 per cent of gum will indicate on hydrolysis by the Clerget method of inversion 1 per cent of sucrose in excess of the amount actually present. In the following tables are given the results of the experiments on the production of gum levan by bacteria in the presence of invertase. The abbreviations used in the tables are as follows:

S.P. = single polarization
I.R. = invert reading
S.C. = sucrose clerget
R.S. = per cent reducing sugars

TABLE 1

The results from Experiment 1 in which invertase was used in the following amounts, 0.1, 0.25, 0.5, 0.75, and 1 cc.

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
Inoculated, 1 cc. bacteria, 1 cc. invertase	23.19	−5.1	−5.2	0.48	−5.0	0.28	0.25	22.00
Inoculated, 1 cc. bacteria, 0.75 cc. invertase	22.00	−4.6	−5.7	1.28	−4.7	0.44	1.06	21.17
Inoculated, 0.5 cc. invertase	22.08	−3.1	−5.5	2.30	−4.1	1.09	1.53	20.00
0.25 cc. invertase	22.08	−2.0	−5.6	3.24	−2.5	1.40	2.28	16.40
0.1 cc. invertase	23.19	−2.5	−5.5	3.91	−3.5	1.04	2.18	16.40
Control	22.14	21.0	−5.7	21.27	−5.4	20.75		

The results in table 1 show that the production of gum by the bacteria is in inverse proportion to the amount of invertase added. In only one case did this inverse relationship between invertase added, and gum produced, fail to exist, and that was in the case of the addition of 0.25 and 0.1 cc. These results agree fairly well with those we obtained in our earlier work (1911 a, 1918) in which we used joint inoculations of yeast and bacteria. It is obvious that in both cases the transformation of sucrose, either by invertase added direct, or secreted by the yeast, tends to deprive the bacteria of the material from which they produce gum levan. The following table shows the results

TABLE 2

Results of experiment in which gum production in the presence of yeast and bacteria was determined

SERIES	CULTURE	ACIDITY—20 CC. USED; CC. N/10 KOH REQUIRED	REDUCING SUGAR	SINGLE POLARIZATION	SUCROSE (CLERGET)	GUM
	Control	0.7		9.3	9.73	
A. (1)	B. IV	4.1	4.25	4.5	5.89	2.69
B. (1)	B. IV and yeast	8.5	7.02	−0.8	2.08	1.47
C. (1)	Yeast	6.6	7.28	−0.6	2.32	
	Control	0.8		9.3	9.44	
A. (2)	B. IV	3.8	6.78	−0.3	1.88	4.85
B. (2)	B. IV and yeast	9.1	2.27	−2.8		0.39
C. (2)	Yeast	5.4	6.25	−4.5		

of one of the earlier experiments in which gum production in the presence of yeast and bacteria was determined.

In table 2 it will be seen that the presence of the yeast in the sucrose solution decreased the amount of gum produced by the bacteria by approximately 50 per cent in one case and almost 90 per cent in another.

In studying the production of gum levan by bacteria in the presence of invertase, it is of great importance to so regulate the amount of invertase added that the inversion will proceed at a regular rate throughout the entire incubation period. If the process is too rapid and glucose and levulose are formed faster than the bacteria can utilize them while in the nascent con-

dition, then the addition of the invertase might be regarded as a disadvantage to the bacteria, even in the light of Greig Smith's theory. In the following experiment the rate of action of the varying amounts of invertase added was measured, and the results are given in table 3.

It will be noted from the results given in table 3 that while the addition of 1 cc. of invertase resulted in an inversion of

TABLE 3

The results of daily analyses showing the rate of action of invertase in experiment 2

SAMPLE	FIRST 24 HOURS		48 HOURS		72 HOURS		96 HOURS		120 HOURS		168 HOURS	
	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.
1 cc. invertase....	14.7	15.87	11.5	13.37	8.3	10.99	6.0	8.5	4.2	7.92	0.3	4.72
0.5 cc. invertase..	17.1	17.82	15.3	16.27	9.7	12.05						
0.25 cc. invertase.	18.3	18.75	17.3	18.13	14.8	16.1	12.6	14.42	10.8	13.07	6.4	8.5
0.1 cc. invertase..	19.0	19.38	18.2	18.65	17.4	18.04	16.1	17.20	15.0	16.3	12.6	14.46

TABLE 4

The results of experiment 2 on the formation of gum levan in the presence of invertase

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
No invertase, 1 cc. bacteria.....	21.9	−8.6	2.9	11.92	−2.1	9.37	3.23	8
0.1 cc. invertase.....	21.9	−3.4	−3.3	2.92	−2.5	1.60	1.67	18
0.25 cc. invertase.....	21.9	−4.0	−3.0	1.96	−2.3	0.7	1.60	17
0.5 cc. invertase.....	21.9	−6.0	−3.2	0.7	−3.1	0.62	0.1	20
1 cc. invertase.....	21.9	6.6	−3.0	0	−3.2			20
Control.....	21.3	19.0	−5.8	19.27	−3.3	20		

sucrose at the rate of 2.5 per cent per day, the addition of 0.5 per cent caused inversion at the rate of 1.5 per cent of sucrose daily, and the smaller additions at correspondingly slower rates. While the addition of 1 cc. would have resulted in the exhaustion of the sucrose before the end of the ten-day incubation period, the smaller additions should have furnished ideal conditions for the maintenance of a constant supply of nascent glucose and levulose. The results of the experiment are given in table 4.

The results in table 4 tend still further to confirm the theory that sucrose, rather than the nascent products of its inversion, is the source of gum levan. If we compare the rate of action of the various additions of invertase as shown in the above table, with those shown in the previous one, where the invertase was acting in the absence of bacteria, we find that the rate of action was much more rapid in the present experiment. For example, where 1 cc. of invertase had been used alone, only about 16 per cent of the sucrose was inverted in one week, while the same amount when used with the bacterial inoculation resulted in the almost complete inversion of the sucrose in a period of ten days. The more rapid action under the latter conditions is due to the production of acids by the bacteria, which makes the condition more favorable for invertase action. The rate of action of invertase in the presence and absence of bacteria was compared in the next experiment, in which larger inoculations of bacteria were used. Instead of a platinum loop, 1 cc. portions of a 48-hour old bouillon culture were employed, in order that the bacteria might have a better opportunity to utilize the invert sugar as rapidly as it is formed. The results are given in table 5.

The accelerative action of the growing bacteria upon the inversion of sucrose by the added invertase is very clearly shown in table 5. While 0.25 cc. of invertase had only inverted about 16 per cent of the sucrose in the 20 per cent solution in one week, the addition of an equal quantity of invertase had inverted 85 per cent of the sucrose when acting in the presence of bacteria for the same length of time. While larger inoculations of bacteria had resulted in an increase in gum production as compared with previous experiments, the ratio between the amounts formed in the presence and absence of invertase was similar to that found in the previous experiments.

The additions of the small amounts of invertase used in the previous experiments could scarcely be expected to inhibit the development of bacteria. However, this possibility was tested in the following experiment, in which the solutions were plated out on a 10 per cent sucrose agar at the end of the incubation period.

TABLE 5

The results of experiment 3 showing the rate of action of invertase in the inoculated and uninoculated sucrose solutions

SAMPLE	48 HOURS			72 HOURS			96 HOURS			144 HOURS			7 DAYS		
	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.
A. invertase only	17.3	−6.3	18.4	15.9	−6.4	17.42	13.8	−5.5	15.20	9.9	−6.2	12.89	9.0	−6.7	12.38
B. 0.25 cc. invertase, 1 cc. bacteria	16.4	−6.3	17.69	12.3	−6.3	14.59	6.2	−5.9	9.71	−2.4	−6.1	3.31	−3.0	−6.3	2.97
C. bacteria only	20.0	−6.4	20.5	19.3	−6.2	19.7	17.7	−5.7	18.3	9.4	−5.8	11.23	6.0	−6.3	9.78

	8 DAYS			10 DAYS			PER CENT R.S.	INVERTASE			PER CENT GUM	SOLIDS
	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.		S.P.	I.R.	S.C.		
A. invertase only	7.0	−6.5	10.74	5.4	−3.5	9.96	12.9		−6.0	9.2		23.02
B. 0.25 cc. invertase, 1 cc. bacteria	3.6	−6.4	2.61	−4.6	−3.3	2.02	20.0		4.4	0.18	2.33	23.05
C. bacteria only	3.4	−6.6	8.01	−8.0	−3.4	5.11	14.9		−0.8	0.74	5.54	22.75

It will be observed from table 6 that the numbers of bacteria in the solutions containing invertase are higher in some cases than where no invertase is present. Since the largest number of bacteria occurred in one case where the largest amount of invertase had been added, the apparent decreases in the other cases where smaller amounts of invertase had been added, cannot be attributed to the inhibiting action of the invertase. Even in the flasks where the bacteria occurred in the smallest numbers,

TABLE 6

The results of experiment 4 showing the development of bacteria and the production of gum levan in the presence and absence of invertase

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	NUMBER PER CUBIC CENTIMETER	S.P.	I.R.	S.C.	PER CENT R.S.	I.R.	S.C.	PER CENT GUM
Control	23.12		20.8	−6.1	21.15	Trace	−5.5	20.77	
Inoculated, 1 cc. bacteria only	22.92	11,800,000	−0.8	−6.0	4.42	15.3	−2.2	1.24	4.03
Inoculated, + 1 cc. invertase	23.12	14,200,000	−2.6	−6.3	3.3	16.9	−1.9	1.21	2.65
Inoculated, + 0.25 cc. invertase	23.16	9,320,000	−4.9	−6.3	1.54	20.0	−5.0	0.57	1.27
Inoculated, + 0.5 cc. invertase	23.46	11,400,000	−5.8	−6.3	1.06	20.4	−5.5	0.1	1.21
Inoculated, 1 cc. invertase	23.49	7,710,000	−6.5	−6.4	0.41	20.6	−5.5	0	0

they were sufficiently numerous to have produced large amounts of gum levan, had other conditions been favorable.

The larger inoculations of bacteria used in this experiment resulted in the production of gum levan in the solution containing 0.5 cc. of invertase, whereas in a previous experiment only a trace of gum had been formed by the bacteria in the presence of that amount of the enzyme.

The results of the previous experiments show that not only does invertase decrease the amount of gum formed, in proportion to the amount of sucrose inverted, but that the remaining

sucrose is utilized to a smaller extent than would be the case if this same amount of sucrose had been available to the bacteria, with no invertase present. This would indicate that the products of the inversion of sucrose hinder rather than aid the bacteria in the production of gum. This fact was observed in our earlier investigations in which various mixtures of sucrose and glucose and levulose were used. The results from those experiments showed that gum production was decreased by the presence of invert sugars, or in other words, that a high ratio between sucrose and invert sugar is most favorable for this type of fermentation. In order to test this point further, the following experiment was conducted. A 20 per cent sucrose solution was inverted by the addition of 4.5 cc. of invertase to 850 cc. of the solution, and allowing it to stand in an incubator at 32°C. for two days. At the end of this period its sucrose content was 2.24, and it was then mixed with an equal volume of an uninverted 20 per cent Greig Smith solution, containing all of the nutrient material in such proportions that after mixing the two solutions, the mixture would conform to the original formula. This solution was then flasked and inoculated, and compared with

TABLE 7

The results of experiment 5 showing the production of gum levan in the presence of reducing sugars

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	PER CENT R.S.	INVERTASE		PER CENT GUM
						I.R.	S.C.	
Inoculated bacteria	22.53	7.8	−6.0	10.97	10.8	−2.86	8.5	3.13
0.25 cc. invertase, inoculated, bacteria	22.82	−5.3	−6.2	1.15	19.5	−4.18		1.55
Half inverted solution, inoculated, bacteria	17.19	+3.2	−4.7	6.38	10.0	−5.06	6.3	
Control sucrose solution	21.70	18.8	−6.0	19.29	Trace	−6.6	19.24	
Control, half inverted solution	17.25	8.4	−4.6	10.26	5.7	−5.2	10.50	
Half inverted solution, inoculated, bacteria 0.25 cc. invertase	17.22	−4.8	−4.7	0.28	16.4	−4.62	0	

a 20 per cent sucrose solution inoculated in a similar manner, and kept under the same conditions.

It will be seen from table 7 that the bacteria formed no gum in the solution containing invert sugar and sucrose, although there was 10 per cent of sucrose present. The relative amounts of gum formed in the sucrose solution with and without invertase were approximately the same as in the previous experiments.

THE PRODUCTION OF GUM LEVAN UNDER VARYING CONDITIONS OF H-ION CONCENTRATION

In the preceding experiments the solution used was approximately neutral in reaction. In previous investigations it had been found that a neutral reaction was most favorable for the gum levan fermentation, but the optimum H-ion concentration had not been determined. In order to determine this point, and to learn whether the optimum for gum production and invertase action coincided, the following experiment was conducted. A 10 per cent sucrose solution (Smith) was prepared and flasked in 200 cc. portions. These were sterilized by the intermittent method, and after the third sterilization and while the contents of the flasks were still warm, measured portions of N/1 NaOH and H_2SO_4 were added. The flasks were then inoculated the invertase added as before. At the end of the ten-day period and analyses were made and the pH values determined on the controls by the potentiometer. The results are given in table 8.

The pH range in this series (table 8) varied from 6.7 to 9.5, and the greatest gum production occurred in the more acid series, when invertase was absent, but in the presence of invertase the amount of gum produced in that series was less than one-tenth of that produced in its absence. In the series containing 0.5 cc. of N/1 NaOH, the gum produced in the presence of invertase was approximately half as great as that produced in the absence of invertase, and as the NaOH was increased a point was reached where the invertase was inactive, and the gum produced in its presence actually greater than where no invertase was added. This beneficial effect of the invertase was probably due to the fact that it protected the bacteria against the action

TABLE 8

The results of experiment 6 showing the development of gum in the presence and absence of invertase in solutions of varying H-ion concentration

SAMPLE	pH	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
						I.R.	S.C.		
Blank, inoculated *B. vulgatus*	7.4	11.49	−1.0	−2.8	1.66	−1.6	0.4	1.98	7.75
Blank, inoculated culture XI.	7.4	11.27	−1.2	−2.8	1.00	−1.6	0.28	1.80	8.29
Blank, 0.5 cc. invertase inoculated, culture XI.	7.4	11.23	−2.8	−3.0	0.38	−2.0		0.48	8.82
0.5 NaOH, inoculated, culture XI.	8.6	11.27	−1.2	−2.4	1.10	−1.6	0.36	1.34	7.86
0.5 NaOH, 0.5 cc. invertase, Culture XI	8.6	11.66	−2.0	−2.4	0.49	−1.6		0.62	8.54
0.5 NaOH, inoculated, *vulgatus*	8.6	11.60	−1.6	−2.8	1.15	−1.6		1.77	8.77
0.5 NaOH, control, 0.5 cc. invertase	8.6	11.66	8.0	−3.6	9.18	−2.4	8.3		2.00
1.2 cc. NaOH, inoculated, culture XI	9.5	11.60	1.6	−3.6	1.81	−1.2		2.29	7.24
1.2 cc. NaOH, inoculated, 0.5 cc. culture XI, invertase	9.5	11.63	−1.6	−3.8	1.98	−1.6		2.51	
1.2 NaOH, inoculated, *vulgatus*	9.5	11.33	9.2	−4.0	10.44	−2.8	9.65	1.00	
1.2 NaOH, control, 0.5 cc. invertase	9.5	11.66	8.8	−3.8	9.97				
2.4 NaOH, inoculated, culture XI		11.63	10.0	3.6	10.72	−3.2	10.6		
2.4 NaOH, 0.5 cc. invertase.		11.37	10.0	3.6	10.72	−3.6	10.9		
2.4 NaOH, 0.5 cc. invertase, control		11.70	10.0	−3.6	10.72	−2.8	10.2		
4.8 NaOH, inoculated, culture XI		11.37	10.0	−4.0	11.06	−2.8	10.2		
4.8 NaOH, 0.5 cc. invertase, control		11.70	9.6	−3.6	10.41	−3.2	10.27		
1.2 cc. N/2 H_2SO_4, inoculated, culture XI	6.7	11.40	−1.2	−3.6	2.12	−1.2		2.57	9.26
1.2 N/2 H_2SO_4, inoculated, *B. vulgatus*	6.7	11.40	−1.6	−3.6	1.81	−1.2		2.29	9.26
1.2 N/2 H_2SO_4, 0.5 cc. invertase, inoculated, culture XI	6.7	11.63	−3.2	3.2	0.41	−1.6		0.17	8.33
1.2 N/2 H_2SO_4, 0.5 cc. invertase, control	6.7	11.66	−2.8	−4.0	1.22	−2.8	0.2	0.129	9.2
Control	6.7	11.40	10.4	2.0	11.3	−0.7	10.5		
Control	6.7	11.70	10.0	2.6	11.06	−0.7	10.24		

of the alkali. In the next experiment a series of solutions were prepared having a pH range from 4.8 to 9.5.[1] The results are given in table 9.

The maximum gum production in this experiment (table 9) occurred in the series with a pH of 7.4. The gum production in

TABLE 9

The results of experiment 7 showing the development of gum in the presence and absence of invertase in solutions of varying pH

SAMPLE	pH	TOTAL SOLIDS BY REFRACTOMETER	S.P.	ACID INVERSION		INVERTASE		PER CENT GUM	PER CENT R.S.
				I.R.	S.C.	I.R.	S.C.		
Blank, inoculated, *B. vulgatus*	7.4	12.44	−2.8	−7.2	3.90	−4.0	1.2	3.43	9.00
Blank, inoculated, *B. vulgatus*, 0.5 cc. invertase	7.4	13.09	−4.4	−4.8	0.67	−1.1	0.33	0.42	12.5
Blank, not inoculated, 0.5 cc. invertase	7.4	13.09	−4.0	−7.2	2.99	−6.4	2.35		11.76
Blank, control	7.4	12.44	9.6	−4.8	11.38	−4.0	10.7		
1.2 cc. NaOH, 200 cc., inoculated, *B. vulgatus*	9.5	12.69	6.0	−5.6	9.30	−4.0	8.08	1.54	2.74
1.2 cc. NaOH, inoculated, culture XI, 0.5 cc. invertase	9.5	12.48	−2.4	−5.6	2.87	−4.0	1.5	1.60	7.14
1.2 NaOH, inoculated, culture XI	9.5	12.48	9.6	−6.4	10.07	−4.4	11.1		
0.5 cc. invertase, 1.2 cc. NaOH, not inoculated	9.5	12.48	8.4	−5.6	8.78	4.8	10.5		1.37
2.4 cc. N/2H_2SO_4, inoculated, culture XI	4.8	12.48	4.4	−4.8	7.40	−4.4	5.25	2.73	3.80
2.4 cc. N/2H_2SO_4, 0.5 cc. invertase inoculated, culture XI	4.8	13.13	−3.2	−4.0	0.91	−4.4	1.21	0.88	12.12
2.4 cc. N/2 H_2SO_4, not inoculated, 0.5 cc. invertase	4.8	12.69	−4.4	−4.0	0	−4.4	0.3		11.76

the series with a pH of 4.8 was greater than in the series having a pH of 9.5. These results show that while the bacteria forming

[1] The writer is indebted to Dr. J. F. Brewster and Mr. W. G. Raines, Jr., of the research Chemical Department, for their kind coöperation in making the pH determinations in the above experiments.

levan from sucrose have a wide range for their activities, their optimum pH does not coincide with the optimum for invertase action. In the presence of invertase, the maximum gum production occurs under conditions where the pH most completely restrains inversion, and the minimum gum production occurs where the conditions are most favorable for it.

THE PRODUCTION OF GUM LEVAN IN RAW AND CLARIFIED SUGAR CANE JUICE

The gum fermentation of cane juices is frequently a problem of considerable economic importance, since it renders the product much more difficult to clarify, and decreases the yields of sugar obtainable from it. An experiment was carried out to determine the relative susceptibility of raw, sulphured, and limed juice to this type of fermentation. A large supply of raw juice was obtained, and divided into two parts, one of which was sulphured to 5.9 cc. (10 cc. of the juice requiring 5.9 cc. of N/10 NaOH to neutralize it). A portion of this sulphured juice was then treated with milk of lime until the acidity had been reduced to 0.5 cc. The original untreated juice made up the third series of the experiment. The pH range was from 3.8 to 6.9. The samples were sterilized on three successive days at 30°, inoculated and incubated as in previous experiments.

The results of table 10 show that the production of gum levan by all three of the cultures used was greater in the limed than in either the raw or the sulphured juice. While the pH of the raw juice was not determined, the average for Louisiana juices is about 5. The acidity of this sample was 1.85 cc., which would indicate a normal juice. From these data it is clear that a clarified juice is much more favorable in its reaction for the production of gum levan than either raw or sulphured juices.

THE SPECIES OF BACTERIA CONCERNED IN THE PRODUCTION OF LEVAN FROM SUCROSE

We have already referred to the fact that Greig Smith (1901, 1902 a), who was one of the first to investigate the bacterial

flora of sugars, gave the name of *Bacillus levaniformans* to the gum forming bacteria that he isolated therefrom. He pointed out the marked similarities between this species and the potato bacillus, especially in regard to the great resistance of its spores to heat, and the characteristic mesentery like growth upon potatoes. The writer in his previous investigation of this sub-

TABLE 10

The results of experiment 7, showing the formation of gum levan in raw, sulphured, and limed cane juice

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	PER CENT R. S.	ACID INVERSION SUCROSE			INVERTASE SUCROSE		PER CENT GUM
			S.P.	I.R.	S.C.	I.R.	S.C.	
1.85 cc. Acid, raw juice, control	16.50	4.00	9.82	4.20	11.09	4.4	11.3	
Raw juice, inoculated, culture XI	15.64	10.4	−0.2	−2.8	2.21	−1.2	1.8	0.6
Raw juice, inoculated, *B. vulgatus*	14.81	10.0	−0.2	−0.4	0.52	−0.6	0.86	
Raw juice, inoculated, potato culture	14.51	7.5	1.4	−4.4	2.65	−1.6	1.6	1.33
Sulphured to 1 cc., 5.9 cc. acid, control	16.96	14.28	2.2	1.61	1.01	1.8	1.2	
Sulphured, inoculated, potato culture	16.96	13.7	2.00	1.80	1.51	2.0	1.7	
Sulphured, inoculated, *B. vulgatus*	16.96	13.2	2.2	2.05	1.85	2.0	1.7	
Sulphured, inoculated, culture XI	16.70	12.7	2.2	1.60	1.01	2.0	1.7	
Limed, 0.5 cc. acid, cold, control	16.70	3.14	10.6	−1.8	11.18	−1.6	11.40	
Limed, inoculated, *B. vulgatus*	16.70	11.4	2.6	2.2	5.72	−1.6	3.37	2.98
Limed, inoculated potato culture	14.85	7.28	1.4	4.8	5.14	−1.6	2.4	3.47
Inoculated, culture XI	16.70	10.6	2.2	−2.2	5.41	−3.2	4.42	1.28

ject found that *Bacillus vulgatus* obtained from the Museum of Natural History of New York City, had the ability to produce gum levan from sucrose, and was in all other respects identical with the cultures isolated from sugars. The other species belonging to the potato group of bacteria, viz., *Bacillus mesentericus-fuscus* and *Bacillus liodermos*, seemed to have little if any such ability. It was found that this levan-forming ability on the

part of *Bacillus vulgatus* could be rapidly increased by frequent transfers in sucrose solutions. After once acquiring this ability, the microörganism retains it apparently, with but little loss. As the gum determinations in the previous investigation were made simply by a measure of the viscosity of the solution, it was decided to repeat them in connection with this investigation, using a more reliable method of gum determination. For this purpose cultures of Bacillus *vulgatus-mesentericus fuscus* and *B. liodermos* were obtained from the Museum of Natural History New York, through the courtesy of Dr. C.-E. A. Winslow. These cultures were used to inoculate flasks containing 200 cc. of a 10 per cent sucrose solution. The inoculations were made from tubes of the same solution, transfers from which were made to fresh sterile solution every forty-eight hours. The cultures that were obtained from sugar were transferred similarly to tubes of sterile bouillon containing no sucrose, or other sugar. These transfers were kept up for a week, at the end of which time a second set of flasks were inoculated. Table 11 shows the production of gum in the first experiment.

The results in table 11 show that the production of gum levan by *Bacillus vulgatus* is much less than that produced by the cultures obtained from sugars. An old culture that had been kept in the form of a spore preparation on a cover glass, produced almost twice as much gum as the former. The latter culture had been repeatedly used in sucrose solutions.

After transplanting the culture as above described, a second inoculation was made with the results indicated in table 12.

From the results of this experiment (table 12), it will be seen that the production of gum by *Bacillus vulgatus* is exactly three times as great as in the former case. There was considerable variation in the effect of the repeated propagation upon the gum-forming powers of the cultures from sugar. As the first experiment might not have been entirely representative, a comparison with the following experiment (table 13) will probably offer a more reliable indication of the effect of these transplantations in a substratum containing no sugars.

TABLE 11

The results of experiment 8, showing the production of gum from sucrose by Bacillus vulgatus when first grown in sucrose solution

CULTURE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	PER CENT R.S.	INVERTASE		PER CENT GUM
						I.R.	S.C.	
VII	13.16	−3.0	−2.15	1.36	5.4	−1.5	0.2	1.39
XI	12.17	−2.8	−1.85	0.95	7.6	−1.4	0.2	0.88
XVI	13.19	−2.2	−2.0	1.65	7.09	−1.1	0.01	2.08
XVII	13.94	−1.6	−2.4	2.76	7.4	−1.2	0.07	3.41
Old culture, *B. vulgatus*	13.19	−3.4	−2.2	1.08	9.0	−1.2	0	1.37
Control	13.84	8.6	−2.6	10.83		−3.3	11.5	
B. vulgatus	13.74	7.6	−2.4	9.70	0.9	−2.0	9.1	0.7
Culture I, old series, spore preparation	14.09	1.0	−2.4	4.73	6.9	−1.5	1.7	3.84
Culture II	13.74	−3.0	−2.2	1.38	8.7	−1.5	0.23	1.46
Culture IV	13.51	−0.8	−2.35	3.3	8.6	−1.7	2.2	1.39
Culture V	14.16	−3.4	−2.2	1.08	8.7	−1.6	0.09	1.25
Culture VI	15.70	−4.0	−2.6	1.29	8.54	−1.6	0	1.63

TABLE 12

The results of experiment 9, showing the increased ability to produce gum levan by Bacillus vulgatus after repeated transfers in sucrose solutions

SAMPLE CULTURE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
Culture I	11.69	+0.6	−4.0	3.8	−1.4	1.65	2.73	7.5
Culture II	10.36	−1.2	−3.2	1.78	−1.2		2.27	7.27
Culture IV	11.60	−1.8	−2.8	0.9	−2.4	0.6		9.0
Culture V	10.36	0.8	−4.8	4.58	−2.8	3.00	2.00	5.8
Culture VI	11.67	−1.2	−4.8	3.04	−2.8	1.45	1.91	7.5
Culture XI	11.08	−0.4	−4.8	3.74	−2.2	1.39	2.98	8.7
Culture XIII	11.45	2.6	−4.4	5.59	−2.8	4.3	1.86	5.9
Culture XIV	11.45	−1.0	−5.4	3.78	−2.0	0.9	3.75	8.5
Culture XVI	11.45	−2.4	−3.6	1.19	−2.0	0	1.51	8.7
Vulgatus	11.01	2.0	−4.8	5.57	−2.8	3.91	2.10	3.5
Mes. fuscus	10.50	7.8	−3.2	8.66	−3.4	8.8		
Old vulgatus	10.43	−1.6	−4.4	2.48	−2.8	1.18	1.65	7.4
Liodermos	10.22	8.6	−3.0	9.14	1.7	9.55		

It is particularly interesting to note from the results in table 13 that six of the cultures isolated from sugars had decreased in their levan-forming power, and one seemed to have lost it entirely. The old *Bacillus vulgatus* produced only one-fourth as much gum as in the first experiment, and no further increase resulted from the transfers of the new *Bacillus vulgatus* culture.

TABLE 13

The results of experiment 10, showing the effect of repeated transfers in sucrose and nonsucrose media upon the ability of Bacilus vulgatus to produce gum levan

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
Culture I, no phosphate	12.85	2.4	−2.8	4.09	−0.8	2.5	2.01	8.5
Culture II, phosphate media	12.95	3.2	−4.0	5.72	1.2	3.48	2.84	9.09
Culture II, phosphate	13.22	1.6	−5.2	5.51	−2.0	2.94	3.26	8.11
Culture II, no phosphate	13.23	2.4	0.9	4.79	−2.0	3.56	1.56	7.33
Culture IV	13.23	1.6	1.1	4.85	−2.8	3.62	1.56	8.69
Culture V	13.23	2.4	−1.2	5.78	−2.8	4.24	1.95	7.84
Culture XI	13.23	1.6	1.1	4.85	−1.2	2.26	3.30	8.69
Culture XIII	13.23	11.2	0.9	11.4	−3.2	11.39		
Culture XIV	12.27	1.6	1.1	5.78	−2.4	3.28	3.17	9.09
Culture XVI	11.66	10.4	1.1	10.53	−2.8	10.39		
Old culture, vulgatus	11.66	2.4	0.9	4.81	−3.2	4.56	0.32	6.94
Culture, *B. vulgatus*	12.31	3.2	1.1	5.32	−1.6	3.82	1.90	7.89
B. liodermos	11.66	11.2	1.1	11.8	−3.2	11.3		
B. mesentericus	11.36	11.2	1.0	11.8	−3.6	11.6		
Culture from potato, 2 transfers	12.56	3.2	1.1	6.08	−2.8	4.84	1.44	7.24
Control	11.67	2.6	1.3	10.49	−3.2	10.6		

From these results one is led to conclude that the production of gum levan by the *Bacillus vulgatus* is a power that is easily acquired and fairly easily lost, until it has become thoroughly established, when it appears that it is held rather tenaciously. This would explain why the old culture of *Bacillus vulgatus* almost entirely lost its gum-producing power, when grown in sugar free media, and why some of the cultures from sugar reacted in a similar way, and why others retained their power

under these same conditions. In addition to being a test of acquiring and losing gum-producing power, this experiment is also a test of the value of phosphates in promoting this type of fermentation. The first four lines of the table show a comparison of gum formation by culture no. 1 and 11 in a solution with and without phosphates. It will be observed that the presence of phosphates greatly promotes gum formation. In the latter part of the table it will be noted that a culture freshly isolated from potato was used, and that it produced 1.44 per cent of gum. The method used to isolate the culture was as follows: A potato was thoroughly cleaned on the outside with a brush. The peelings were then put in a 300 cc. Erlenmeyer flask containing some sterile sucrose solution. The flask was then placed in the incubator, and after growth had taken place, transfers were made to tubes of the same medium. Sucrose agar plates were then made and a pure culture obtained.

It will be noted that neither in this nor in the previous experiment was any gum formed by *Bacillus liodermos* or *Bacillus mesentericus-fuscus*. This is approximately the same conclusion that we reached in the previous investigation to which we have referred.

In order to determine whether the acquisition of gum-producing power is more rapid when the bacteria are transferred to sucrose solutions of higher densities than the 10 per cent that we had been using, a comparison was made between a 10 per cent and a 20 per cent solution.

The cultures used in this experiment (table 14) were the *Bacillus vulgatus* and the culture isolated from a potato, which we had used in the preceding experiment. It will be noted that the gum-producing power of the former culture was much greater after being grown in the 20 per cent sucrose solution. The other culture showed a slightly greater gum-forming power after being grown upon the 10 per cent solution. The results of our earlier investigation had very strongly indicated that the gum-forming power is acquired more rapidly by these species of bacteria, when they are grown in concentrated sucrose solutions.

The conditions for acquiring this property are to be found in cane sugar factories. The gum-forming bacteria are brought to the mill in the trash and particles of soil adhering to the cane. When the cane is crushed they are carried into the juice, and their spores survive the temperatures attained during the boiling and evaporating of the juice, and the crystallization of the sirup.

TABLE 14

The results of experiment 11, showing the relative rate at which gum-forming ability is acquired in 10 and 20 per cent sucrose solutions

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	PER CENT GUM	INVERTASE		PER CENT R.S.
						I.R.	S.C.	
Control	12.16	11.0	−3.2	11.02		−2.0	10.43	
Control	11.82	10.8	−2.8	10.54		−2.0	9.9	
Inoculated, *B. vulgatus*, 10 per cent sucrose solution	11.50	−0.8	−2.4	1.38	1.25	−1.2	0.39	7.35
Inoculated, *B. vulgatus*, 20 per cent sucrose solution	11.82	−0.8	−4.0	2.70	2.92	−1.2	0.4	7.8
Potato culture, 10 per cent sucrose solution	11.83	−0.4	−5.2	3.99	4.35	−1.2	0.4	7.1
Potato culture, 20 per cent sucrose solution	11.58	+0.4	−5.0	4.4	3.49	−1.6	1.65	6.94
B. mesentericus, 10 per cent sucrose solution	11.07	10.2	−2.6	9.91		−3.6	10.7	
B. mesentericus, 20 per cent sucrose solution	11.58	10.8	−2.9	10.62		−3.2	10.92	
B. liodermos, 10 per cent sucrose solution	11.58	9.8	−2.6	9.61		−2.8	9.8	
B. liodermos, 20 per cent sucrose sulution	11.58	10.6	−2.6	10.22		−2.8	10.4	

The great resistance of the spores of these bacteria to heat, and their ability to withstand the osmotic pressure of the high density sirups and molasses to which they are subjected, fit them admirably for this environment. The potato group of bacteria are enabled to withstand this osmotic pressure, owing to the fact that they have permeable (Fischer, 1903) cell membranes, and hence their cell walls are not easily plasmolyzed. It is on this

account that we find some of these species capable of producing gum levan in sucrose solutions of 50 to 60 per cent. We conclude therefore that the soils surrounding cane sugar factories will gradually become inoculated with potato bacteria which have acquired the ability of forming gum levan from sucrose, owing to the practice of returning the filter press cake to the soil. The final molasses from cane factories, when fed to the stock on the plantation, would probably result in infecting the manure with these gum-forming species, which would again find their way back to the soil in the manure. Hence there will be a continuous cycle of acclimatization and distribution of these organisms on a sugar plantation.

THE PRODUCTION OF GUM LEVAN FROM SUCROSE IN ITS RELATION TO THE POTATO GROUP OF BACTERIA

We have already referred to the fact that in morphological and physiological characteristics the bacteria isolated from sugars are, with the exception of the ability to form gum levan from sucrose, identical with the potato species. The potato group of bacteria comprise the following species: *Bacillus vulgatus* (Trevisan), (*Bacillus mesentericus vulgatus* (Flugge) and potato bacillus of various authors), *Bacillus mesentericus fuscus* (Flugge), (*Bacillus mesentericus* (Trevisan) and *Bacillus liodermos* (Flugge), (Chester, 1901)). No data are given by Chester on the behavior of these species in sucrose solutions, although a closely related species *Bacterium panis* (*Bacterium mesentericus, panis-viscosus*), which was first isolated from stringy dough is said to produce a viscous fermentation of bread dough. Migula (1897) classes *Bacillus liodermos* as identical with the gum bacillus of Loeffler. Chester (1903) who studied *Bacillus mesentericus*, mentions only the production of acid in sucrose solutions. However, he also describes a culture of *Bacillus mesentericus fuscus* obtained from the Bacteriological Laboratory of the Johns Hopkins University, which he named *Bacillus subtilis* var. *viscosus*. The following description of the growth of this species on agar very strikingly suggests the species we have under investigation. "*Agar cultures have the same thick glassy viscid appearance and the growth*

can be drawn out into long threads, or behaves under a needle-like thick mucus." Lohnis (1913) refers to the activities of certain gum-forming varieties of the potato bacillus, in the viscous fermentation of the diffusion juice in beet sugar factories. From a review of the literature on the physiological characteristics of the potato group of bacteria it is apparent that but little attention has been paid to their ability to induce the gum fermentation in sucrose solutions. In view of this fact it can be readily understood how easy it would be to overlook this characteristic of these species, particularly since it seems to be largely an acquired ability. Under these circumstances it is very probable that many of the gum-forming species of bacteria have been given specific names, when they are really only derived types of the potato group of bacteria, which have been developing for successive generations upon sucrose solutions.

CONCERNING THE NATURE OF THE TRANSFORMATION OF SUCROSE INTO GUM LEVAN

The results of this investigation have shown that sucrose is the substance from which gum levan is formed, by the bacteria that we have been studying. It has been shown further that sucrose can supply the needs for levan-forming bacteria only when it exists as such in the culture solution. The presence of invertase decreases gum formation in the exact proportion in which it hydrolyzes the sucrose present. A study of the tables shows also that, in the absence of invertase, the amount of gum formed is always in proportion to the sucrose that has been utilized by the bacteria in the solution. In other words, we have here a true fermentation in which definite products are formed, and which is not to be regarded as the result of an abnormal condition of the bacterial cells. We have already referred to the fact that the species of bacteria concerned in the gum levan fermentation do not have a demonstrable capsule. This indicates that the gum is formed extracellularly. Evidence of the existence of an extracellular gum-forming enzyme in connection with this type has been presented by Beijerinck (1910), who succeeded in producing gum levan extracellularly by

the use of extracts prepared from these same species. The gum fermentation of sucrose is, in the light of our present knowledge, of a two-fold advantage to the species of bacteria inducing it; enabling them in the first place to transform sucrose into an assimilable form, and in the second place reducing the osmotic pressure of the sucrose solution, rendering the environment more favorable for their continued activities. For these purposes this fermentation is of greater advantage to the microörganism than the secretion of invertase would be, because the inversion of the sucrose would offer no protection to the bacteria from the osmotic action of the solution, while its conversion into gum would reduce it in proportion to the amount of this colloid formed. It is not easy to explain how this transformation of the disaccharide sucrose into the polysaccharide gum takes place. Browne (1912) has suggested the possibility of the action being a combined assimilative and enzymic action, the sucrose being assimilated into a higher saccharide, which is broken up by an enzyme into glucose and levan.

CONCLUSIONS

The formation of gum levan from sucrose does not depend upon the action of invertase, as implied in Greig Smith's theory of the nascent glucose and levulose origin of the gum, but on the contrary is entirely prevented by the rapid inversion of sucrose by this enzyme.

The action of invertase not only decreases the production of gum levan to the extent to which it inverts sucrose, but retards the production of this gum from the sucrose remaining in the solution.

Decreasing the ratio of sucrose to invert sugars decreases the production of gum levan.

The optimum pH for the development of gum levan is between 6.7 and 7. The relative differences in the amounts of gum produced by the bacteria in the presence and absence of added invertase, was greatest in the solutions whose pH values were nearest the optimum for the action of invertase, and least where the pH values were least favorable for invertase action.

A culture of *Bacillus vulgatus* was found to have the ability to form gum levan from sucrose, and by repeatedly growing it in the presence of a suitable sucrose solution this ability was greatly increased. It is concluded from our study of the species of levan-forming bacteria occurring in sugars, that they are all derived types from the potato group of bacteria. Many of the similar species described in the literature as distinct species are thought also to be derived types.

The gum fermentation of sucrose is believed to be a distinct type of fermentation, probably acquired as a means by which an organism secreting no invertase may convert the unassimilable disaccharide sucrose into assimilable forms, and into products whose combined osmotic pressure value is lower than that of the original sucrose. In this manner the material for supplying the energy for the organism is provided, and the environment, if its osmotic value is too high, is rendered more favorable for the continued growth and development of the organism.

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A READILY CULTIVABLE VIBRIO, FILTERABLE THROUGH BERKEFELD "V" CANDLES, VIBRIO PERCOLANS (NEW SPECIES)

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During the investigation of certain electrocapillary effects of importance in filtration through laboratory filters, (Mudd 1923), it became desirable to have a filterable living organism whose surface electric potential could be studied in connection with its filterability. Search was accordingly begun, at the suggestion of Prof. S. B. Wolbach, and in October, 1921, an undescribed and easily cultivable vibrio was isolated in the filtrate from hay infusion made from fresh water from near Boston. This vibrio passes readily through Berkefeld "V" candles impervious to *Erythrobacillus prodigiosus* and *V. comma*.

The point of zero potential difference between this vibrio and its medium unfortunately corresponds to a concentration of hydrogen ions so high as to be incompatible with the organism's normal activity, so that it has proved unsuitable for determining the effect on filterability of reversing this interfacial potential. On the other hand, the new vibrio has proved so useful in a variety of ways for testing other factors in filtration that its description seems merited.

This organism is a short, actively motile, comma-shaped to straight, rod with rounded ends, occurring singly or in short chains. There is typically one polar flagellum, though frequently two or three are present. The flagella are rarely bipolar; in length they range from 3 to 7μ. With the fuchsin encre stain of Nicolle and Morax they are readily demonstrated.

In length this vibrio ranges from 0.5 to 2.5 μ, the large majority being 1.5 to 1.8 μ long, as determined by measurement of photomicrographs and by the use of a filar micrometer. The thickness is about 0.3 to 0.4 μ.

In young cultures (eighteen to twenty hours) filamentous forms are frequently found, some of which are chains of organisms, but in which, in other instances, subdivision is with difficulty, or not at all, detectable. The filaments are typically long slender spirals; in smears, however, they often appear straight or with only slight curvature.

The organism is Gram-negative. It stains with the ordinary laboratory dyes.

There are no spores, and usually the protoplasm, as seen by dark-field illumination and in stained specimens, appears homogeneous, except for one or more fine granules in an occasional organism. However, when a culture some days old, containing many individuals, which have lost motility and presumably vitality, is examined with dark-field illumination many of the non-motile organisms appear to consist of brightly shimmering, coarse granulations. This same coarse granulation has been occasionally noted, in hay infusion too acid for optimum growth, even in a majority of the motile individuals of the culture. Subcultures on media with proper reaction return to the normal appearance.

Very occasionally, in old (ten to twenty days) acid hay infusion cultures, "involution" forms similar to those of *V. comma* are found; large globoid forms which stain faintly, spoon-shaped and clubbed organisms, and forms which show a banded appearance with carbol-fuchsin. However, the appearance of these markedly atypical "involution" forms is not very common—not nearly so common as in the case of *V. comma*.

Our most satisfactory cultures of this organism have been grown on hay infusion of reaction near neutrality. Under optimum conditions, the medium is diffusely clouded in six to ten hours, and in twenty-four hours a pellicle forms, sometimes tough and whitish, sometimes merely a scum which is faintly iridescent. After two to three days a slimy deposit settles out

at the bottom of the tube. When the tube is swirled, this deposit comes up in the form of a coarsely twisted rope, looking much like an inverted water-spout.

In bouillon and in Witte's pepton growth is similar to that in hay infusion, but rather less abundant.

Litmus milk is unchanged, and no pellicle forms, though growth occurs.

At first the vibrio grew with difficulty on nutrient agar; after one or two generations, however, growth became profuse. The typical twenty-four hour colony is round, convex and slightly flattened on top, 1 to 2 mm. in diameter, with entire margin. The consistency of the colonies is mucoid, and they tend to become confluent. By reflected light they appear bluish-white and glistening; viewed by transmitted light under low magnification, they are yellowish and finely granular. Colonies five to six days old, where there is sufficient room for growth, reach 6 to 7 mm. diameter and show a distinct central boss (pl. 1). The deeper agar colonies are lenticular.

There is no liquefaction of gelatin. The character of the colonies is the same as on agar. In a gelatin stab, while growth is good on and near the surface, it is very faint and scattered along the line of the stab. Growth is not so abundant on Loeffler's blood serum as on either agar or gelatin, and there is no digestion of the medium. On potato the colonies are small, white, and slimy. There is no diastatic action on starch. Nitrates are not reduced, nor is gas formed. No indol is produced.

There is no fermentation of the following carbohydrates: glucose, lactose, sucrose, maltose, mannitol, adonitol, rhamnose and xylose. The most favorable temperature for growth is about 30°C.

The vibrio is actively motile, both in fluid media and in suspensions of young cultures from solid media. Examination of eighteen to twenty-four hour hay infusion cultures shows most of the individuals free-swimming, relatively large and slow-moving; a few small groups of agglutinated organisms are to be seen. In older cultures (several days old) agglutinated masses are much more in evidence and the free-swimming organisms are smaller,

a few even coccoid, and motility appears much swifter and more vigorous. In hay infusion cultures, weeks or months old, motility may be almost absent, although subculture proves viability still present.

Progression with rotation on the long axis is the characteristic mode of motion with the vibrio. A greater or less degree of precession, or "wobbling" of the long axis, is also to be seen in a large number of the organisms. The combination of these movements in the small, swiftly moving forms produces a strong impression of a progressive undulation passing down the organisms, which, however, is apparently illusory, since the long, slow-moving, filamentous forms present in young hay infusion cultures can be seen to retain their shape during motion, and to be without "notable" flexibility. During division, when the daughter individuals are separated by an obvious constriction, their axes may be quite movable with reference to each other.

The direction of motion is reversible. An individual may often be seen to dart forward, then backward, then forward again in a slightly different direction in a way suggestive of the avoiding reaction of paramecium.

A filterable spiral form, *Spirillum parvum*, has also been described by von Esmarch (1902). To this, our organism in some ways appears similar. However, *Sp. parvum* does not grow on either potato or milk. Old agar colonies have a violet tinge. Moreover, it grows very slowly, rendering fluid media turbid only after eight or ten days. Among the larger forms of filterable organisms should be mentioned the spirochaetes, both parasitic and free-living, whose passage through the Berkefeld filter Wolbach (1915) has demonstrated, and the closely allied Leptospirae (Noguchi, 1918, 1919). In view of the distinctive morphological and cultural characteristics of the new organism and its filterability, we propose the name *Vibrio percolans*.

An agglutinating serum for *V. percolans* can be produced in guinea pigs by intraperitoneal injections of 2 cc. of a twenty-four bouillon culture of the organism every other day for fourteen days. This serum, if allowed to act for twenty-four hours at room temperature, agglutinates *V. percolans* in dilutions up to

1:750. Normal guinea-pig serum has no agglutinating action on the organism. *V. percolans* agglutinating serum has no effect on *V. comma*, *Sp. Finkler-Prior*, or *Sp. metchnikoffi*.

Table 1 summarizes the results obtained in determining the thermal death point of *V. percolans*. The cultures used were twenty-four hours old in the bouillon of $(H^+) = 1.3 \times 10^{-7}$ (pH = 6.9). Approximately one and a half minutes was required to raise the culture to the given temperature. The results were read after forty-eight hours incubation of the subcultures at 30°C.

TABLE 1

DEGREES CENTIGRADE	TIME														
	½ minute	1 minute	1½ minutes	2 minutes	2½ minutes	3 minutes	4 minutes	5 minutes	6 minutes	8 minutes	9 minutes	12 minutes	15 minutes	16 minutes	20 minutes
56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
54	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
53	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0
52	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
51	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates that the subcultures made at the time specified gave a growth of the vibrio; 0 indicates negative subculture.

The organism is fairly resistant under various conditions. It survived after at least eight days at 0°C. in bouillon. At laboratory temperature one glucose broth culture, plugged merely with cotton, was viable after two months. One of the original subcultures, kept at room temperature on a paraffin plugged agar slant, is alive after seven months. Subcultures made from hay infusion cultures kept only with cotton plugs at laboratory temperature for more than six months in several instances have been positive, as is illustrated by the following protocols.

Large test tube (3.5 cm. diameter) of hay infusion inoculated December 11, 1921 with *V. percolans*. Kept in laboratory with cotton plug until June 29, 1922. Then had evaporated to a little less than

half original volume. Short, medium and filamentous forms of *V. percolans* to be seen under dark field, a very occasional individual motile. Subculture in twenty-four hours swarming with vibrios of high motility.

Test tube (2.5 cm. diameter) of hay infusion inoculated November 26, 1921. By June 29, 1922, had evaporated to a little less than a quarter original volume; opaque, chocolate color; under dark field, heavy suspension of vibrios, an occasional one still motile. Subculture positive.

Test tube (2 cm. diameter) inoculated October 11, 1921. By June 29, 1922, evaporated to a little less than quarter original volume occasional motile vibrio present. Subculture positive.

This vibrio is not pathogenic for white mice, white rats, wild rats (*Rattus norwegicus*), guinea-pigs, and rabbits, either by subcutaneous or intraperitoneal injection or by ingestion. One of us (S. W.) swallowed 5 cc. of a twenty-hour broth culture without detecting any effect.

We would call attention to the photomicrographs illustrating this paper and the following paper taken by means of the apparatus for ultra-violet photomicrography kindly placed at our disposal by the late Prof. H. C. Ernst.

The light source is the 2800 Å. line of a magnesium arc. By means of an apparatus for mechanical focussing, devised by Dr. W. T. Bovie, it is possible to focus accurately on the bacteria, using for illumination the harmless green portion of the magnesium spectrum, and then to change directly to the proper focus for ultra-violet light. In this way, the organisms are subjected to ultra-violet light during only the actual exposure of the plate (three to five seconds).

In order to be sure the organisms were not killed by this exposure, the photographed preparations, which were made in 0.5 per cent agar in saline, were rubbed up in sterile broth and plated out. In every case a heavy growth was obtained.

Both from the point of view of theory (V. Sabine (1906) and Ernst and Wolbach (1906)) and of practical results, it is believed that the ultra-violet microscope gives more nearly the actual structure of the living bacterial cell than does any other method of direct observation.

POWER OF LOCOMOTION OF V. PERCOLANS AS COMPARED TO THAT OF V. COMMA AND E. PRODIGIOSUS

In correlation with the study of motility as affecting filterability described in the paper subsequent, investigation was made of the passage of *V. percolans*, *V. comma* and *E. prodigiosus* through a coarse-pored filter without aid by any pressure head and dependent solely upon their own powers of locomotion and growth. The following modification of the method of Carnot and Garnier (Besson, 1914) has been used:

A U-tube, 14 mm. inside diameter, with arms 20 cm. long, has a plug of glass wool placed in the bottom of one arm. Above this is placed a layer of 60/120 quartz sand (average diameter of grains 0.23 mm.), acid-washed, about 10 cm. in depth. Hay infusion or nutrient bouillon is poured in, filling both arms to a level one centimeter above the sand, the arms plugged with cotton, and the whole autoclaved. When ready to inoculate, 2 to 3 cc. of the medium is removed from above the sand with a sterile pipette, and replaced with an equal amount of a suspension of the organism to be tested. The tubes are then incubated. Appearance of a faint clouding in the uninoculated arm of the tube indicates the end point. By dividing the time in hours from inoculation to clouding of the sand-free arm by the depth in centimeters of the sand layer, the approximate average time required by the organism to pass 1 cm. of the sand is obtained.

In the experiments each organism was placed under its optimum conditions of growth. *E. prodigiosus* and *V. comma* were grown at 37°C., the former in bouillon and the latter in pepton bouillon. *V. percolans* was grown at 30°C. in faintly alkaline hay infusion. The slightly lower temperature of cultivation may have been a serious handicap to *V. percolans* in comparing its rate of passage with that of *V. comma;* if so *V. percolans* was also handicapped in the filtration experiments in which its temperature was again somewhat lower.

V. comma showed consistently greater speed in effecting passage than did *V. percolans*. The optimum rates obtained were as follows:

ORGANISM	TIME FOR PASSAGE 1 CM. OF SAND
V. comma (Rosebank strain)	1 hour 48 minutes
V. percolans:	
Strain V_{18}	2 hours 24 minutes
Strain V_{19}	2 hours 29 minutes
Strain V_{29}	2 hours 30 minutes

E. prodigiosus[1] did not pass the sand in the six experiments performed: three experiments were of one week and three of two weeks' duration.

A culture of *V. percolans* has been deposited in the Army Medical Museum, Washington, D. C., whence any worker interested may obtain a culture.

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[1] The *E. prodigiosus* strain used was from the Medical School stock. Transplants on certain media showed limited motility. In bouillon or suspension from plain agar for instance a few organisms were motile. A culture on blood agar was somewhat more motile. A culture on wet potato showed greatest motility. Of the organism in the fluid at the bottom of the potato tube a considerable number, possibly five per cent, were motile. Motility in hay infusion cultures was not with certainty observed. Such individuals as were motile on any media, with the possible exception of the potato fluid, were quite obviously less vigorous and rapid in their movements than the cholera or percolans vibrios.

EXPLANATION OF PLATE

PLATE 1

FIG. 1. LIVING TWENTY-FOUR HOUR HAY INFUSION CULTURE OF V. PERCOLANS.

Ultra-violet light, magnesium line of λ2800 Ångstrom units. Five seconds exposure. ×1120.

FIG. 2. PLAIN AGAR COLONIES FIVE DAYS OLD

Natural size. Drawn as seen by reflected light

FIG. 3. PLAIN AGAR COLONIES

Transmitted light; low magnification

FIG. 1

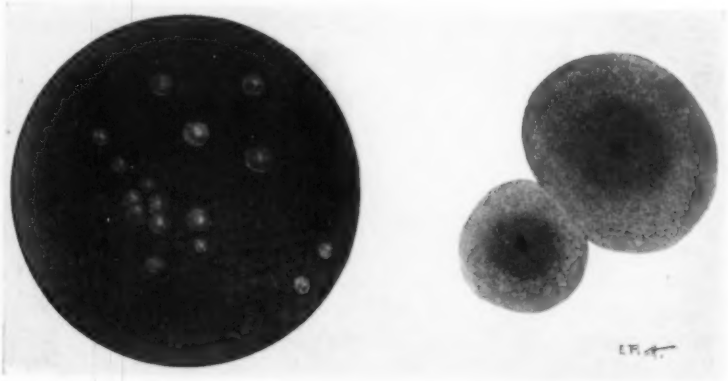

FIG. 2

FIG. 3

(Mudd and Warren: A readily cultivable vibrio.)

THE PENETRATION OF BACTERIA THROUGH CAPILLARY SPACES

I. MOTILITY AND SIZE AS INFLUENCING FILTERABILITY THROUGH BERKEFELD CANDLES

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The contact surface of the pores of a Berkefeld filter and the water bathing them is the site of an electric potential difference, an ordinary Helmholtz electric double layer, the solid walls taking the negative, and the fluid the positive, charge (Mudd, 1923). If a solution of a "basic" dye, such as methylene blue, is drawn through the filter, electro-positive color ions are taken out by the electro-negative filter capillary walls, and the first portions of the solution may be partially or completely decolorized; more prolonged filtration results in saturation of the filter pores, a colored filtrate and colored wash water. Acid dyes, such as eosine, on the contrary may be filtered without demonstrable change in color; the electronegative color ion does not adhere to the negative capillary walls. The filter is "basophilic."

The filterable microörganism described in the preceding paper, *Vibrio percolans*, in ordinary culture media, was found to be negatively charged with reference to the suspending medium: migration in an electric field, studied with the aid of a microcataphoresis chamber, was toward the anode. The hope was entertained that by acidifying the medium a hydrogen ion concentration could be reached at which the vibrio would still maintain its activity but would carry the positive component of the electric double layer, its filterability thus being suppressed. However vibrios are known to be especially susceptible to acid, (Besson, 1914), and a hydrogen ion concentration of about

5×10^{-6} (pH = 5.3) or above was found to inhibit the motility of *V. percolans*, although insufficiently high to reverse the direction of the surface potential difference. Therefore the question whether or not a filterable microörganism will be found whose filterability can be suppressed by reversal of the interfacial potential with consequent adsorption on the negative filter walls remains an open one.

It was observed, however, that when cultures of *V. percolans* were filtered in media with hydrogen ion concentrations greater than 5×10^{-6} (pH = 5.3) no vibrios grew out in media inoculated with the filtrate; this raised the question whether the motility of a culture could be a determinative factor in its filtration. It has subsequently been found that the passage of *V. percolans* through Berkefeld V candles can be prevented by suppressing its motility with acid, with ether and chloroform, or with cold.[1]

SUPPRESSION OF FILTERABILITY OF V. PERCOLANS BY ACID

The reaction of the culture of *V. percolans* was at first adjusted by adding a sufficient amount of buffer solution of the desired hydrogen ion concentration. This method in the one experiment tried resulted in stoppage of motility and a negative filtrate; adjustment was made to H-ion concentration of 6×10^{-5} (pH = 4.2). Uncertainty as to possible toxic effect of the buffer ions led to abandonment of this method in favor of a safer one, namely, the washing out of the CO_2 from the culture with a stream of hydrogen and the addition of N/10 HCl until the desired reaction was reached. The hay infusion culture, usually at an initial H-ion concentration not far from 5×10^{-7} (pH = 6.3), by bubbling hydrogen through it for a half to three quarters of an hour could be brought to an approximately stable reaction around 1×10^{-8}, (pH = 8). Acid was then added as desired. The CO_2 could be bubbled from the culture somewhat more quickly if it was kept a little acid by addition of HCl during

[1] By "prevented" is meant prevented at least for intervals slightly more than sufficient to allow the motile control culture to inoculate a filtrate under similar conditions. It is possible, however, that a more prolonged filtration of the culture with motility inhibited might have resulted in inoculation of the filtrate.

the process (Henderson, 1920). The reaction was followed throughout with a Bovie direct-reading potentiometer (Bovie 1915–1916). The condition of the vibrios was followed by observing samples microscopically at frequent intervals with dark field illumination. No change was observed when the cultures were rendered slightly alkaline or acid, but when the H-ion concentration rose above 5×10^{-6} (pH = 5.3) motility became obviously impaired, and above 1×10^{-5} (pH = 5) was practically stopped, save in one culture which had been grown in a medium more acid than usual and in which the H-ion concentration had to be raised to about 4.5×10^{-5} (pH = 4.35) in order to stop locomotion. When motility was observed practically to have ceased the culture was filtered, and, in the seven experiments performed, proved uniformly negative for vibrios; the control filtrates from unacidified cultures were regularly positive, i.e., contained vibrios.

The complete experimental procedure was typically as follows:

The suspension filtered was always a mixed hay infusion culture of *V. percolans* and a weakly motile strain of *Erythrobacillus prodigiosus*. The vibrios were oridinarily the outgrowth in sterile hay infusion tubes of the filtrate from the previous control experiment. The culture was strained if necessary through coarse meshed cloth to remove flocculi and sediment. It was then put in a titration vessel of the Bovie potentiometer, the CO_2 washed out and the hydrogen ion concentration adjusted at from 1 to 5×10^{-6} (pH = 6 to 5.3). Motility of the vibrios at this reaction was still normal as seen by dark field observation. The culture was then divided into two equal parts, the first of which was rendered sufficiently acid to stop locomotion and then filtered, with negative pressure (suction), starting at zero and increasing the pressure difference on the two sides of the filter to several hundred millimeters of mercury; the filtrate was inoculated into sterile hay infusion tubes. Various amounts of the residual unfiltered acid culture were next inoculated into similar sterile hay infusion tubes as controls. The fact that both *V. percolans* and *E. prodigiosus* uniformly grew out in the latter tubes proved that the culture had not been

sterilized by the short acid exposure. The second half of the original culture, with motility slightly if at all impaired, was then passed through the same filter used for the acid filtration, and the filtrate inoculated into the sterile hay infusion tubes; *V. percolans* regularly grew out of these tubes. *E. prodigiosus* was regularly absent in all filtrate and filtrate-inoculated tubes.

The filters used in the experiments of this paper were Berkefeld "V" (No. 3, 2½ inches long by ⅝ inch in diameter) with wall thickness of approximately 4 mm. The filtration lasted only two to six minutes, as shown in the tables. Twenty-five to 60 cc. of filtrate were drawn through the candle for each experiment. The unfiltered culture was invariably turbid and the filtrate clear; several examinations of filtrate specimens under the dark field microscope immediately after filtration did not serve to detect vibrios, though these ordinarily grew out in twenty-four hours or less when the filtrate was positive.

From all analogy it seems certain that the acidification of the culture decreased the potential difference at the filter-fluid and bacteria-fluid interfaces. The fact that seven experiments in which motility was stopped with acid gave clearly negative filtrates, whereas when motility was inhibited by other agencies (see below) only five cultures showed complete suppression and five partial suppression of filterability, may possibly be of significance; decreasing the charges on bacteria and filter wall may have allowed the molecular adhesion, "stickiness," to act more effectively in holding the bacteria to the filter capillary walls. This suggestion is advanced at this time, however, purely as a speculation.

As to possible changes in the size of the organisms with acidification of the media, none was ever observed under the dark field, though a more sensitive measure than mere inspection might with profit be employed. However we would expect such a change if it occurred to have been in the direction of a shrinking rather than a swelling, as acid was added and the organisms were thereby brought toward their point of zero potential difference against the medium (Loeb, 1922) just as hydration of gelatin particles decreases as they are brought toward their isoelectric point.

SUPPRESSION OF FILTERABILITY OF V. PERCOLANS BY NARCOTICS

The coincidence of the H-ion concentration at which the motility of *V. percolans* ceased with that at which filterability was lost made it desirable to stop motility by other means. This was done with narcotics, and filterability was again found to be coincidently suppressed.

For each pair of experiments an actively motile culture was as before divided into two halves; one-half was shaken with a small amount of ether or chloroform and allowed to stand until dark field examination showed that locomotion had practically ceased. This was then filtered, the filtrate inoculated into sterile hay infusion tubes and other tubes inoculated with the unfiltered narcotised culture as a control of its viability. The untreated half of the original culture was then passed through the same filter and the filtrate inoculated into sterile tubes.

The residue of the narcotised unfiltered culture was allowed to stand in open cylindrical vessels until the anaesthetic had evaporated. Subsequent dark field examinaton showed the culture swarming with motile vibrios indicating, as did the inoculated control experiments mentioned above, that the chloroform and ether narcotised but did not kill the majority of the vibrios.

SUPPRESSION OF FILTERABILITY OF V. PERCOLANS BY CHILLING

Finally confirmation of the belief that suppression of the motility of the vibrio could inhibit its filtration through Berkefeld V candles was obtained by chilling the cultures. For each pair of experiments an actively motile culture was put in the ice box surrounded by ice water, and with melting ice in the culture vessels themselves. The filter was also chilled. One half of the culture was filtered at about 0°C.; the other half was allowed to warm for from twenty odd to seventy odd minutes and was then passed through the same filter. Vibrios regularly appeared in tubes inoculated with the latter filtrate, but were absent in some or all of the tubes inoculated with the filtrate from the filtration at 0°. Control inoculations of the culture at 0° into

TABLE 1

Effect of reaction of the medium on filtration of V. percolans

EXPERIMENT NUMBER	FILTER NUMBER	H-ION CONCENTRATION	MOTILITY	FITRATION TIME	FILTRATION PRESSURE	RESULT	REMARKS
				minutes	*mm. Hg.*		
V_1	1	6×10^{-6}	Almost stopped	6	0–286	–	Na-acetate acetic acid buffer used
V_2	1	1×10^{-6} 1×10^{-7}	Normal	5	0–295	+	*E. prodigiosus* control showed filter tight
V_3	1	2.3×10^{-8}	Practically stopped	4	0–155	–	CO_2 bubbled out in this and later experiments
V_4	1	Ca. 1×10^{-7}	Normal	4½	0–195	+	
V_5	2	7.4×10^{-6}	Little if at all impaired	3½	0–225	+	New filter used
V_6	2	7.9×10^{-8}	Little if at all impaired	3	0–245	+	Acid culture from V_3 rendered alkaline and filtered
V_7	2	Not acidified	Normal	3-4	0–245	+	
V_8	2	Not acidified	Normal	7	0-145	+	
V_9	2	Not acidified	Normal	3	0–235	+	*E. prodigiosus* control showed filter tight
* V_{10}	3	7.2×10^{-6}	Impaired	3½	0–255	–	New filter used
* V_{11}	3	9.5×10^{-7}	Normal	4	0–225	+	*E. prodigiosus* control showed filter tight
V_{12}	Vibrios apparently killed by twenty odd minutes exposure to H-ion concentration about 1.5×10^{-8}. No growth in control tubes						
V_{13}	3	3×10^{-8}	Normal	2½	0–195	+	*E. prodigiosus* control showed filter tight
V_{14}	3	$1.2\text{-}1.3 \times 10^{-6}$	Much impaired	2½	0–245	–	
V_{15}	3	2×10^{-6}	Little if at all impaired	5½	0–245	+	*E. prodigiosus* control showed filter tight

V_{16}	4	1.6×10^{-5}	Much impaired	6½	0–109	−	New filter used
V_{17}	4	3.6×10^{-6}	Little if at all impaired	5	0–109	+	*E. prodigiosus* control showed filter tight
V_{18}	4	1.7×10^{-6}	Practically stopped	3½	0–225	−	
V_{19}	4	3.2×10^{-6}	Slightly impaired	3½	0–225	+	
V_{20}	Results inconclusive because of bad culture medium						
V_{21}	Results inconclusive because of bad culture medium						
V_{22}	5	4.5×10^{-6}	Practically stopped	3¼	0–145	−	
V_{23}	5	3.2×10^{-6}	Little if at all impaired	2	0–145	+	*E. prodigiosus* control showed filter tight

* Experiments bracketed performed with two halves of same culture as above described.

TABLE 2

Effect of narcotics on filtration of V. percolans

EXPERIMENT NUMBER	FILTER NUMBER	APPROXIMATE PER CENT NARCOTIC ADDED*	MOTILITY	FILTRATION TIME	FILTRATION PRESSURE	RESULT	REMARKS
				minutes	*mm.Hg.*		
V_{26}	3	Chloroform 0.28 per cent added; not all dissolved	Much reduced	2¼	0–200	Negative all tubes	Filter had been cleaned with Na_2CO_3 and water since using for V_{18}
V_{27}	3	Untreated	Active	2½	0–180	Positive 4 tubes, negative 1 tube	
V_{28}	3	Ether 2.7 per cent	Practically stopped	5½	0–100	Negative all tubes	
V_{29}	3	Untreated	Active		0–105	Positive all tubes	*E. prodigiosus* control showed filter tight
V_{30}	2	Ether 6.5 per cent added	Practically stopped	3¼	0–220	Negative all tubes	
V_{31}	2	Untreated	Active	3	0–220	Positive all tubes	*E. prodigiosus* control showed filter tight
V_{32}	5	Ether 3.9 per cent added	Practically stopped	2	0–270	Negative 5 tubes, positive 1 tube	
V_{33}	5	Untreated	Active	2	0–270	Positive all tubes	*E. prodigiosus* control showed filter tight
V_{34}	3	Ether 3.2 per cent added	Greatly reduced	3½	0–335	Negative 3 tubes, positive 2 tubes	
V_{35}	3	Untreated	Active	3½	0–335	Positive all tubes	

* The percentage of narcotic tabulated is probably somewhat greater than that actually present in the cultures because of partial evaporation during additions of the ether and examinations of the cultures.

sterile hay infusion tubes at room temperature gave uniformly positive results.

The organisms regained active motility with surprising quickness on rewarming after chilling. Preparations could scarcely be got on the dark field microscope without showing motility, and within a few seconds locomotion was active.

ATTEMPTS TO FILTER VIBRIO PERCOLANS THROUGH N AND VIBRIO COMMA THROUGH V CANDLES

Inspection under the dark field microscope of living cultures of *V. percolans* and of *V. comma* impresses the observer with the general similarity of the two organisms but also with two peculiarities of *V. percolans* which are doubtless significant in determining filterability. These are, first, the frequency of highly motile, small, almost coccoid forms in *V. percolans* cultures, and, second the greater slenderness of *V. percolans*. Filar micrometer measurement has given as the average diameter of one hundred each of the organisms, *V. comma*, 0.46μ; *V. percolans*, unselected, 0.35μ; *V. percolans* (smaller individuals) 0.31μ, and *E. prodigiosus*, 0.31μ. The range of diameters in the *V. percolans* vibrios measured was 0.28μ to 0.51μ.

Doubtless in correlation with these characteristics is the fact that *V. percolans* under normal cultural conditions regularly gives a positive filtrate with Berkefeld V candles (though uniformly negative in six attempts at passage under similar conditions through three N candles) whereas *V. cholerae* is not a filter passer. Four unsuccessful attempts were made to pass *V. comma* through three Berkefeld Vs which had been shown before and after the attempted *V. comma* passage to be pervious to *V. percolans*.

DETECTION OF V PERCOLANS IN GROUND DOWN FILTER SECTIONS

Berkefeld filters, as is well known, are made of baked kieselguhr (diatomaceous earth). By microscopic examination of scrapings from a dry filter the fine irregular silicious granules

TABLE 3

Effect of chilling on filtration of V. percolans

EXPERIMENT NUMBER	FILTER NUMBER	PREPARATION	MOTILITY	FILTRATION TIME	FILTRATION PRESSURE	RESULT	REMARKS
				minutes	*mm. Hg.*		
V_{36}	6	Chilled	Greatly reduced	2½	0–312	Negative all tubes	
V_{37}	6	Chilled and rewarmed	Active	2½	0–310	Positive all tubes	
V_{38}	6	Chilled	Much reduced	3¼	0–240	Negative 4 tubes, positive 1 tube	
V_{39}	6	Chilled and rewarmed	Active	3¼	0–240	Positive all tubes	*E. prodigiosus* control showed filter tight
V_{40}	7	Chilled	Much reduced	3½	0–300	Negative all tubes	
V_{41}	7	Chilled and rewarmed	Active	2¾	0–250	Positive all tubes	*E. prodigiosus* control showed filter tight
V_{42}	8	Chilled	Reduced	4½	0–355	Negative 2 tubes, positive 3 tubes	
V_{43}	8	Chilled and rewarmed	Active	1¾	0–342	Positive all tubes	
V_{44}	7	Chilled	Reduced	6	0–400	Negative 5 tubes, positive 1 tube	
V_{45}	7	Chilled and rewarmed	Active	3¼	0–395	Positive 4 tubes, negative 1 tube	

may be seen. Study of filter sections ground down to microscopic thinness shows a matrix of the fine granules containing at intervals spaces or lacunae of much greater size, i.e., a few to more than a hundred micra. For photomicrographs, see von Esmarch (1902) and Hofstädter (1905).

It has been the judgment of most authors who have considered the point that these lacunae could not afford a continuous passage through the filter wall and that a part, and of course a critical part, of the path of particles traversing the filter must be through the matrix or actual granular filter substance itself. This view has not been universally accepted, however, so that it seemed necessary to study the distribution of the vibrios in the filter.

A used V filter, no. 8, was again immersed in a vibrio culture and a filtration pressure maintained for a few minutes. It is to be emphasized that the bacteria were carried into the filter by filtration of only a few minutes duration and with filtration pressure of a few hundred millimeters of mercury. They were not allowed to "grow" into the filter. Absolute alcohol was drawn through to fix the freshly deposited vibrios. Fuchsin (acid) was drawn through for several hours in an attempt to stain the organisms satisfactorily; this later proved to have been unsuccessful. The filter was dehydrated and balsam in xylol was drawn through by suction and then allowed to seep through for a day or more. The balsam-impregnated filter was allowed to harden for a couple of weeks in the incubator. Rings were then cut with a knife and ground down with a coarse stone. A ring was mounted in balsam on a glass slide and final reduction to microscopic thinness carried out by prolonged grindings on fine stones. The balsam was removed by soaking in xylol, the xylol allowed to evaporate, and the organisms stained *in situ* with carbol fuchsin (acid) or methylene blue. The filter fragment was finally washed, dried and mounted in xylol balsam and studied with oil immersion.

The result is quite conclusive (see plate 2). The organisms may be seen in a stained coagulum on the surface and extending down into the superficial lacunae, and in among the granules of the matrix. By focussing with the fine adjustment the trans-

parent granules may be brought into focus above and below the vibrios, proving the latter to be in the capillary interstices between the silicious grains of the actual filter substance.

In plate 2 the organisms are readily discernible, a few in focus, others below and above the focal plane. No lacunae are shown in the photomicrograph, though they are present in other fields of the section. Intense illumination was used for the photomicrograph in order to give definition to the vibrios and the detail of the filter structure was thereby sacrificed. A few silica granules can, however, be made out.

THE INTERGRANULAR DIAMETERS AND RELATIVE POROSITY OF DIFFERENT BERKEFELD FILTER TYPES

The pore size of a number of Berkefeld filters has been estimated by the methods of Bechhold (1908). Compressed air was forced through the filters and the pressure in millimeters of mercury at which streams of bubbles began to issue from six or more pores was substituted as p in the formula:

$$D = \frac{4\beta \times 760}{p \times 1.933 \times 10^5}$$

β is the capillary constant of water at the temperature of the determination. D is the diameter in millimeters of the narrowest portion of the pores from which the bubbles emerge, i.e., it is an estimate of the average value of the spaces between the granules of the actual filter substance.

By using a small escape valve in the air line and a mercury manometer air pressure could be maintained within a few millimeters of any desired level. The values given in table 4 are in each case the average of several observations; they agree within a few mm. for any clean filter, but in used filters may vary by as much as 40 mm. Hg.

The variations in the mean values for the three types of filters, V. N. and W, is seen to be small; moreover it is in the wrong direction, i.e., the V's or most porous, appear to have the smallest intergranular spaces. These differences are thus probably fortuitous.

It may be pointed out that some of the used filters whose pores had been contaminated with dyes and proteins emitted bubbles at much lower pressures than the new ones, apparently indicating larger intergranular diameters. A similar effect was produced by mixing alcohol with the distilled water wetting the filters. It is thus evident that Bechhold's formula gives too high values for pore size in the presence of substances lowering the surface tension of the water in the pores.

TABLE 4

FILTER	SIZE	CONDITION	PRESSURE	CALCULATED DIAMETER	AVERAGE DIAMETER FOR FILTER TYPE
	inches		*mm. Hg*	*μ*	*μ*
V (10)	$2\frac{1}{4} \times \frac{5}{8}$	New	520	0.414	
V (11)	$2\frac{1}{4} \times \frac{5}{8}$	New	567	0.380	
V (16)	$2\frac{1}{4} \times \frac{5}{8}$	New	585	0.373	0.38
V (17)	$2\frac{1}{4} \times \frac{5}{8}$	New	581	0.376	
V (18)	$2\frac{1}{4} \times \frac{5}{8}$	New	588	0.371	
N (X)	$2\frac{1}{4} \times \frac{5}{8}$	New	466.5	0.461	0.45
N (XI)	$2\frac{1}{4} \times \frac{5}{8}$	New	493	0.437	
W (1)	$2\frac{1}{4} \times \frac{5}{8}$	New	410.5	0.524	
W (2)	$2\frac{1}{4} \times \frac{5}{8}$	New	450	0.478	0.43
W (X)	$2\frac{1}{2} \times 1$	New	592	0.369	
W (XI)	$2\frac{1}{2} \times 1$	New	636	0.343	
V (6)	$2\frac{1}{4} \times \frac{5}{8}$	Used	588	0.366	
N (1)	$2\frac{1}{4} \times \frac{5}{8}$	Used	617.5	0.349	
N (2)	$2\frac{1}{4} \times \frac{5}{8}$	Used	468	0.460	
N (VIII)	$2\frac{1}{2} \times 1$	Used	287	0.761	
W (IX)	$2\frac{1}{2} \times 1$	Used	307.5	0.710	

Tested by the second method, namely that of determining the rate of flow of distilled water through the filters as a function of the pressure head, the filters of course showed themselves more porous in the order V > N > W. Plotting pressure against rate of flow, the points fall along straight lines passing very nearly through the origin. The slopes indicated the following rates of flow for three new $2\frac{5}{8} \times \frac{5}{8}$ inches filters:

V (10), 0.45 cc. per second per 100 mm. Hg pressure head
N (X), 0.21 cc. per second per 100 mm. Hg pressure head
W (1), 0.125 cc. per second per 100 mm. Hg pressure head

The filters used thus evidently differed in the number and size of the gross pores or lacunae rather than in the size of the intergranular spaces.

With the foregoing conclusion in mind further attempts were made to filter *V. percolans* through N filters, as follows:

Approximate volume filtered, 150 cc.; filtration pressure, 0–400 mm. Hg; result negative
Approximate volume filtered, 125 cc.; filtration pressure, 0–500 mm. Hg; result negative
Approximate volume filtered, 575 cc.; filtration pressure, 0–700 mm. Hg; result negative
Approximate volume filtered, 480 cc.; filtration pressure, 0–744 mm. Hg; result negative

The passage of filterable organisms through the filter wall is evidently then greatly facilitated by the lacunae, even though one critical part of the passage has to be through the fine interstices of the granular filter substance.

DISCUSSION

Porous filters of the Berkefeld type, as of course is well known, have shown themselves of much practical utility in freeing solutions of thermolabile substances from bacteria, in separating the ordinary bacteria from the "filterable viruses" (Wolbach, 1912), in purifying water, and in other ways. The majority of these filters under the usual conditions of short duration of filtration and moderate pressure give filtrates sterile for most known microörganisms. However, the familiar fact that the microörganisms penetrate the filter if contact of the culture with it is sufficiently long-continued, especially if suitable nutrient material is at hand in the filter pores (Hofstädter, 1905), would seem to indicate that the filter owes its tightness to the tortuosity and length of the passage to be traversed as well as to its narrowness, rather than to an absolute excess in all cases of the smallest dimensions of the microörganisms over the smallest diameters of the pores they must pass through.

Adhesion also doubtless plays a part in holding back the bacteria (Bechhold, 1918). In dark field examination of cultures of *V. percolans* in films between cover-glass and slide the vibrios are found adherent to the upper or lower glass surfaces in increasing numbers as time goes on. Often a vibrio can be seen to swim against the cover glass and stick there; occasionally an organism will stick to the cover glass only at one point and thresh about in the medium with this contact as a fixed point. With *E. prodigiosus* this stickiness doubtless plays even a more considerable part, for colonies on solid agar are extremely adherent and when scraped with a platinum loop come off in long strings. A fluid culture of *E. prodigiosus* can be distinguished from one of *V. percolans* also by the stringing out of the former when it is filtered through cheese-cloth or even when a platinum loop is withdrawn from the prodigiosus culture.

The "effective pore size" of a bacteria-tight filter has been defined by Rosenthal (1908) as "the narrowest diameter which is present in each of the extraordinarily many porous passages, at least in one place; it becomes, if we consider layers of one and the same mass increasing regularly in thickness, smaller, at first rapidly, then more slowly." He estimates the effective pore size as between 0.5 and 2μ.

Schmidt (1910) from the fact that he was able to filter *Ps. fluorescens-liquefaciens* but not staphylococci and Gram-negative diplococci, whose diameter was about 0.8μ, gives 0.2μ and 0.8μ as upper and lower limits of the effective pore size of Berkefeld filters. "One will certainly not be far from the true value, if he assumes a mean value of about 0.5μ." Assuming that the smallest quartz grains visible in ground-down sections of Berkefeld filters approximate close-packed spheres, he calculates the actual diameter of the finest pores between them as about 0.3 to 0.4μ.

Bechhold (1908) has calculated the diameter of pores of filters by the minimum pressure able to force air through. He gives the mean pore diameters of the larger pores of a new chamberland F filter as 0.23 to 0.41μ.

Our own estimates on Berkefeld filters are given above.

In view of these estimates it is of interest that under the circumstances of our filtrations motile *V. percolans* passes through Berkefeld V but not Berkefeld N candles and *V. comma* through neither. The measured diameters of one hundred unselected individuals of *V. percolans* vary from 0.28 to 0.51μ, with an average value of 0.35μ. Selecting the smaller individuals, the mean diameter found was 0.31μ. The average diameter of one hundred unselected cholera vibrios was 0.46μ.

The mechanics of the situation determining sterility or nonsterility of the filtrate from any given bacterial culture are evidently then such that under ordinary circumstances we may expect bacteria-free filtrates, but in the presence of any circumstances exceptionally favorable to passage through the filter, we anticipate a seeding of the filtrate. This expectation the literature shows indeed to be fulfilled.

The favoring circumstances may be small size alone e.g., the globoid bodies of poliomyelitis (Flexner and Noguchi, 1913), of 0.2μ average diameter, the coccoid bodies described by Foster as a cause of coryza (1917), typically 0.2 to 0.3μ in diameter, *Bacterium pneumosintes* (Olitsky and Gates, 1921, 1922), 0.15 to 0.3μ in length, a half to a third as large in diameter and the minute Gram-negative anaerobes isolated from human throats by Olitsky and Gates (1922), and probably the coccoid bodies described by MacCallum and Oppenheimer in vaccine lymph as about one-tenth the size of ordinary streptococci (1922).

Or small size and motility may combine to produce filterability, e.g., *Spirillum parvum* of v. Esmarch (1902), diameter 0.1 to 0.3μ, and the filterable spiral organisms and the protozoan of tap water discovered by Borrel (1903):

> Ceux qui passent le plus ordinairement sont des vibrions très polymorphs dont certains sont à la limite de la visibilité, reconnaissable à leur cil unique. Ces formes sont celles qui passent à tràvers les pores de la bougie, puis, dans la culture, il se développe des vibrions de dimensions variable, quoique cette culture paraisse tout a faire pure. Dans d'autre cas, ce sont des formes spirillaires qui passent et la culture montre des très longs filamentes grêles, invisible à l'état frais, assez semblables aux spirilles de la fièvre recurrente, mais plus courts. . . . .

Dans ce même milieu, j'ai obtenu, par filtration, la culture pure d'éléments très particuliers, que je considère comme appartenant au groupe des protozoaires, et que j'ai désigné sous le nom de *Micromonas mesnili*. Ce sont très ordinairement des éléments ovoïdes allongés de $\frac{1}{4}\mu$ de largeur sur 3 à 4μ de longeur, munis de deux cils trepus, plus gros que des cils de bacteries, plus rigide . . . . etc.

A clear case of the determinative value of motility is the passage of motile *V. percolans* through Berkefeld Vs and the failure to pass of non-motile percolans.

Slenderness, motility and flexibility may all be complementary factors in enabling organisms to pass the filter, e.g., the filterable spiral organisms of Wolbach and Binger, *Sp. elusa* (1914) and *Sp. biflexa* (1915), and *Leptospira icterohaemorrhagiae* (Noguchi 1919), diameter 0.25μ, and Noguchi's *Leptospira icteroides* (Noguchi, 1919), 0.2μ in diameter.

Again slenderness, motility and flexibility may not suffice and specially favorable pressure conditions may be require in addition, e.g. the experience of Todd and Wolbach (1914), who conclude that:

Sp. duttoni in an infective form can be forced through a Berkefeld filter by pressures of over 50 pounds to the square inch.

Sp. duttoni cannot be filtered through a Berkefeld filter in an infective form by atmospheric pressure.

Similarly Hofstädter, in an admirable work on the penetration of bacteria through the finest capillaries (1905), was able to force bacteria through capillaries under a filtration pressure of 50 to 100 atmospheres which would not permit their passage under pressures of two to three atmospheres or without a pressure head.

It is a pleasure to acknowledge the many courtesies of the late Prof. H. C. Ernst, of Profs. S. B. Wolbach and W. T. Bovie and assistance on a number of points of Mr. Shields Warren and Emily B. H. Mudd.

SUMMARY

Cultures of *Vibrio percolans*, grown in hay infusion whose reaction was approximately neutral, when brought to a hydrogen-

ion concentration of 5×10^{-6} (pH = 5.3) or above rapidly lose motility. Such non-motile cultures gave negative filtrates with Berkefeld V candles through which motile vibrios regularly passed.

Similarly the filterability of *V. percolans* under the conditions of our experiments was suppressed completely five times and partially five times by inhibiting motility with ether, with chloroform or by chilling.

In all cases the viability of the non-motile culture, at least for the duration of the experiment, was proved by positive subcultures. Growing with *V. percolans* in the cultures which were filtered was a feebly motile strain of *E. prodigiosus*. This organism did not appear in the filtrates whether positive or negative for *V. percolans*.

It is thus shown that when a culture is drawn through an irregular capillary bed under circumstances in which a few organisms may or may not pass through, motility may be a critical factor in determining passage. Motility doubless aids passage both in purely mechanical ways and by combating the tendency of the organisms to adhere to the pore walls.

V. percolans did not in ten attempts made pass through Berkefeld N candles. Attempts to pass *V. comma* through three Berkefeld V's shown before and after to be pervious to *V. percolans* were likewise unsuccessful. *V. comma* repeatedly showed itself able to pass by its own powers of growth and locomotion through a layer of ten centimeters of quartz sand in shorter time than *V. percolans*. Its inability to traverse Berkefeld filters is therefore not referable to inferior motility but to slightly larger size than *V. percolans*. The average diameters found for cholera vibrios (unselected) was 0.46μ, for *percolans* (unselected), 0.35μ, for *V. percolans* (smaller individuals), 0.31μ, for *E. prodigiosus* (unselected), 0.31μ.

Estimation of the diameters of the intergranular spaces of Berkefeld filters by Bechhold's method of forcing air through gave the following average values:

V type, 0.38μ; N type, 0.45μ; W type, 0.43μ. The differences are interpreted as fortuitous.

Estimation of porosity by determining the rate of filtration of water at various pressures shows the V type the most and the W the least porous. The following filtration rates with distilled water for $2\frac{5}{8} \times \frac{5}{8}$ inch candles were obtained:

V, 0.45 cc. per sec. per 100 mm. Hg pressure head
N, 0.21 cc. per sec. per 100 mm. Hg pressure head
W, 0.125 cc. per sec. per 100 mm. Hg pressure head

The differences in porosity are then evidently a matter of the relative size and numbers of the gross pores rather than of the intergranular spaces.

Sections of a Berkefeld V candle used for filtration of *V. percolans* have been ground down to microscopic thinness and the vibrios stained and demonstrated in the interstices between the silicious granules of the filter substance. The intergranular diameters are therefore of critical importance in determining the penetrability of the filters by bacteria.

That the relative numbers and sizes of the gross pores or lacunae may also be of critical significance is shown by the successful passage of motile vibrios through V filters and their failure to pass N's.

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PLATE 1

FIG. 1. E. PRODIGIOSUS, TWENTY-FOUR HOUR AGAR CULTURE IN 0.5 PER CENT AGAR MOUNT

Five inches, 1.9 mm., 7 oc. × 1120 diameters. Ultraviolet light, λ = 2800 Å, Mg electrodes.

FIG. 2. V. CHOLERAE, TWENTY-FOUR HOUR CULTURE

Conditions same as above

FIG. 3. V. PERCOLANS, TWENTY-FOUR HOUR CULTURE

Conditions same as above

Photomicrographs by Mr. Shields Warren

PLATE 2

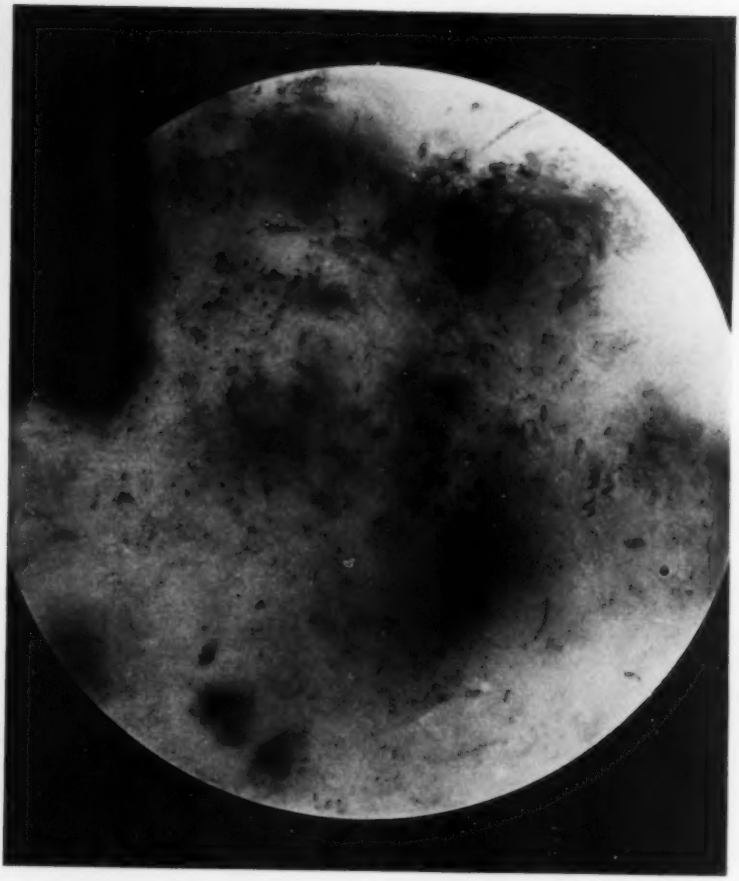
GROUND DOWN SECTION OF BERKEFELD V FILTER WITH VIBRIO PERCOLANS STAINED IN SITU WITH METHYLENE BLUE

A few vibrios in focus, others below and above the focal plane. Darker zone along left margin of field deeply stained coagulum at surface of filter. Oil immersion. × 1000 diameters. Photograph by Prof. S. B. Wolbach.

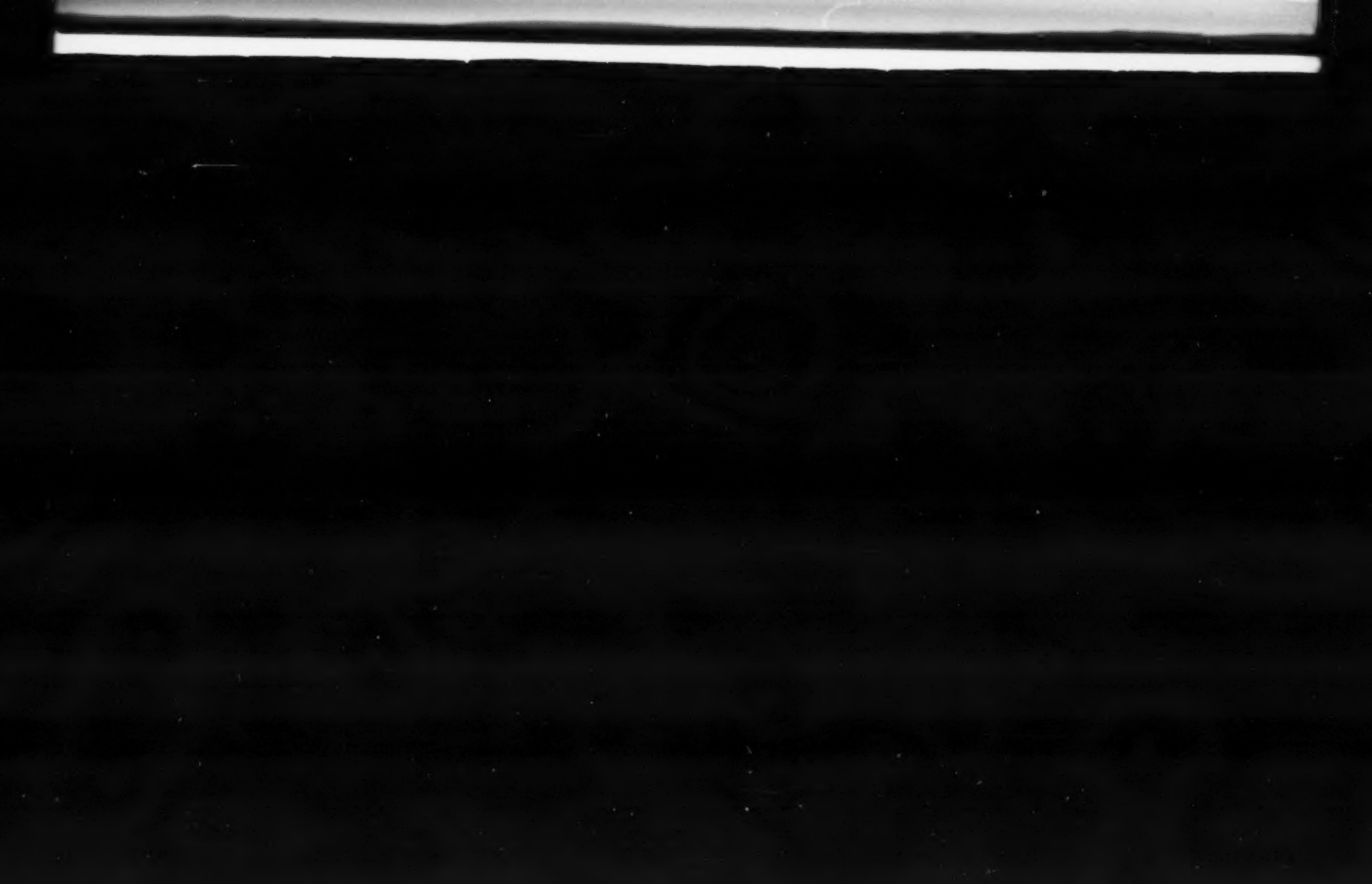
FIG. 1

FIG. 2

FIG. 3



(Mudd: Penetration of bacteria.)



PHYSIOLOGICAL STUDY OF AZOTOBACTER CHROOCOCCUM. I

INFLUENCE OF VITAMINE B (?) AND NUCLEIC ACID ON AZOTOBACTER

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INTRODUCTION

This paper deals with a preliminary investigation in regard to the influence of vitamine B (?)[1] and phyto-nucleic acid[2] upon the growth and nitrogen-fixing power of Azotobacter,[3] an investigation which was undertaken in conjunction with our major problem of soil fertility now in progress.

EXPERIMENTAL

The influence of different concentrations of vitamine B (?) and phyto-nucleic acid on the growth of Azotobacter

These substances were tested for their accessory action by adding them in varying concentrations to Ashby's solution of pH 7.2. First 1 per cent sterile solutions of these substances were made and varying amounts added to sterile, double concentrated Ashby's solution, in volume equivalent to one-half the final volume. Finally the solution was made up to the desired volume by adding sterile water. The medium, after being

[1] Vitamine B(?), yeast vitamine which was prepared by the Harris Laboratory, Tuckahoe, New York, and shown to contain water-soluble vitamine B, in highly concentrated form.

[2] Phyto-nucleic acid, prepared from yeast cells by the Will Corporation, Rochester, New York.

[3] Azotobacter, A4 of Prof. D. H. Jones of O. A. C., Guelph, Canada.

tested for sterility, was inoculated with 0.05 cc. of a ten-day-old culture of Azotobacter in Ashby's solution.

Table 1 indicates that the addition of these substances at 1:10,000 or higher concentration seems to have a similar stimulating effect on growth. No difference in degree of stimulation in concentrations above 1:10,000 is apparent so far as the visual observation of turbidity is concerned.

TABLE 1

The influence of varying concentrations of vitamine B(?) and phyto-nucleic acid on the growth of Azotobacter

CONCENTRATIONS	GROWTH AFTER FIVE DAYS AT 25°C.	
	Vitamine B(?)	Phyto-nucleic acid
1:500	++	++
1:1,000	++	++
1:10,000	++	++
1:100,000	+	+
1:1,000,000	+	+
Ashby solution (plain)	+	+

Rate of acceleration of growth

An attempt was made to record the rate of acceleration of growth by these two substances in 1:10,000 concentration.

From table 2 it is apparent that the stimulation is manifest within forty-eight hours, and is very marked at the fifth day. Here again it may be noted that the two substances studied are behaving very similarly.

TABLE 2

Rate of acceleration of growth

MEDIA	24 HOURS	48 HOURS	96 HOURS	120 HOURS	240 HOURS	360 HOURS
Ashby + vitamine B(?)	−	+	+	++	++	++
Ashby + phyto-nucleic acid	−	+	+	++	++	++
Plain Ashby	−	−	+	+	+	+

+, moderate visible growth; ++, indicates abundant visible growth.

Quantitative determination of growth of Azotobacter and nitrogen fixed

At the end of ten days, the number of organisms was counted by plating on Ashby's agar and the total nitrogen was determined.

Table 3 indicates the quantitative increase in the number of Aztobacter cells and the amount of nitrogen fixed. It is apparent that there is a slight difference in the stimulation by vitamine B (?) and phyto-nucleic acid, the former appearing to be more effective than the latter. Also it is interesting to note the parallelism between the growth and the total amount of nitrogen fixed.

TABLE 3

Growth of Azotobacter and nitrogen fixed

MEDIA	NUMBER OF ORGANISMS PER CUBIC CENTIMETER		N PER 100 CC.		GAINED
	Initial	After ten days	Initial N	After ten days	
		millions	*mgm.*	*mgm.*	
Ashby + vitamine B(?)	19,000	1,550	0.2	15.0	14.8
Ashby + phytonucleic acid	19,000	1,250	0.2	13.0	12.8
Plain Ashby	19,000	450	0.2–	5.7	5.5

DISCUSSION

The vitamine B (?) used in the investigation was prepared by the Harris Laboratory and was claimed to be a highly concentrated vitamine, water-soluble-B (fraction II) described by Osborne and Wakeman.[4] It is stated that the fraction II contains 7.5 per cent of nitrogen, or 4.46 per cent of the total yeast nitrogen, or 31.8 per cent of the nitrogen of yeast extract. Consequently the concentration of 1:10,000 which was used in the investigation contained a very small amount of nitrogen, approximately 0.0075 mgm.

The influence of this preparation on *Saccharomyces cerevisiae* was determined by the author as well as by his students in the physiology class and an accessory effect similar to that reported

[4] Jour. Biol. Chem., 1919, vol. 40, no. 2, 383.

by Bachman[5] was observed. Recently animal tests on this preparation were carried out by Bailey and his co-workers[6] and a marked accessory influence was noted.

The phyto-nucleic acid (1:10,000) which was used in this investigation contains approximately 0.016 mgm. of nitrogen.

When the experimental data recorded previously are considered in the light of the facts stated, it seems to be apparent that these two substances exert an accessory influence other than the supply of a very small quantity of food substance.

It is not the purpose of this paper to convey the idea that vitamine B (?) and phyto-nucleic acid are similar in substance. The experimental data, however, indicate plainly that both of these substances have a stimulating influence on *Azotobacter chroococcum*.

CONCLUSIONS

The water-soluble-B (?) vitamine and phyto-nucleic acid exert marked stimulating influence on Azotobacter A4 in regard to its growth and fixation of nitrogen.

[5] Ibid., 1919, vol. 39, no. 2, 235.
[6] Bull. 240, Connecticut Agr. Exper. Station, 1922, 44–47.

THE ENZYMIC CONTENT OF BACTERIAL SPORES[1]

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That bacterial spores are lacking in enzymic activity is a natural deduction from the fact that the spore is a resting stage in the life history of the organism. However, the work of the Kopeloffs[2] on the demonstration of active enzymes in mold spores suggests the possibility of active enzymes in bacterial spores. It is the purpose of this paper to record some preliminary experiments on the possibility of demonstrating active enzymes in bacterial spores. After our work was begun the following statement was found in Effront's "Biochemical Catalysts in Life and Industry" (p. 312):[3]

> In this connection we must also take into consideration the observation of Effront, according to which the bacterial spores, attenuated either by an antiseptic or by heat, show themselves the more productive of enzymes the more difficult their germination. Under certain conditions and in the presence of antiseptics the spores may produce an intense secretion of enzyme in a liquid without, however, arriving at germination.

Up to the present time we have been unable to obtain Effront's original paper and so we do not know what data he had to support his statement.[4]

[1] Published by the Permission of the Director of the Michigan Agricultural College Experiment Station.

Read at the Detroit meeting of the Society of American Bacteriologists, December 29, 1922.

[2] "Do mold spores contain enzymes?" M. Kopeloff and L. Kopeloff, Jour. Agric. Research, 1919, **18**, 195–209.

[3] "Biochemical Catalysts in Life and Industry." Jean Effront. Trans. by S. C. Prescott. First Ed., John Wiley and Sons, 1917.

[4] Between the time of submitting this paper for publication and the reading of the proof Effront's original article ("Sur l'Action Chimique des Spores," Jean Effront. Le Moniteur Scientifique, Queensville, Feb., 1907, Liv. 782, pp. 81–87) has been found and studied; his data agree in every respect with ours.

In the present work suspensions of washed spores were added to certain substrates in the presence of antiseptics and observations and chemical determinations were made for the purpose of detecting chemical changes in the substrates.

Twelve cultures of aerobic, spore-forming bacteria were used. The names of the species will not be given at the present time because a positive identification has not been made. The cultures were purified by plating and then sown upon the surface of agar in Kolle flasks and allowed to incubate for five and one-half months at room temperature under a bell jar. The growths were then scraped off with a sterile, bent glass rod and suspended in sterile physiological salt solution. These spore suspensions were centrifuged for forty to fifty minutes, which resulted in precipitating the spores and leaving the few vegetative rods in the supernatant liquid which was pipetted off. This process of washing was repeated a second time and then the spores were made into a uniform suspension in physiological salt solution. In this connection it is worthy of note that before the washings, the growth contained small numbers of vegetative rods which had failed to sporulate, but that after washing, such vegetative rods could not be found. Apparently the spores were appreciably heavier than the rods. Possibly also the latter were dead since these cultures were several months old. From 1 to 2 cc. of these spore suspensions were used to inoculate the various substrates.

The following is a description of the substrates used with the results secured with each substrate:

OXIDASE TESTS

1. Two cubic centimeters of spore suspension added to 10 drops tincture of guaiac (1 gram resin in 60 cc. of 95 per cent alcohol). All negative.

2. Two cubic centimeters of spore suspension added to 10 drops of a 1 per cent alcoholic α-napthol solution. All negative.

3. Two cubic centimeters of spore suspension added to 10 drops of a 1 per cent aqueous solution of p-phenylene-diamine hydrochloride. All negative.

4. Indophenol test on 2 cc. of spore suspension. All were slightly positive, while controls were negative.

REDUCTASE TESTS

One-half cubic centimeter of a methylene blue solution (5 cc. saturated alcoholic solution of methylene blue + 195 cc. of water) was added to 10 cc. water. Added 1 cc. of spore suspension. All tubes negative after five hours at 45 to 48°C.

CATALASE TESTS

A sterile fermentation tube was filled with 1 per cent hydrogen peroxide. Two cubic centimeters of spore suspension were added. All 12 organisms produced an evolution of gas, 4 of them being very active. Boiling of the spore suspension greatly reduced the activity, though in no case was it prevented entirely.

LIPASE TESTS

One cubic centimeter of the spore suspension was added to 100 cc. of a butterfat emulsion (made by emulsifying pure butterfat with gum acacia and water, adding 1 per cent formalin and sterilizing in flasks in the autoclave). After incubating for two weeks in a dark room, the acidity in 10 cc. of an uninoculated control was compared with that in the inoculated flasks. To 10cc. of the emulsion was added 50 cc. of neutralized water and then 50 cc. of a neutralized mixture of alcohol-ether (1 : 1). One-tenth normal alcoholic potassium hydroxide was used for titration and rosolic acid was used as the indicator. Eleven of the cultures were clearly negative, while one of them gave an increase of 0.4 cc. of tenth normal potassium hydroxide over the control (0.8 for control, 1.2 for culture).

CASEINASE TESTS

To 350 cc. of sterile skim milk was added 2 cc. of the spore suspension, enough chloroform to form a distinct layer on the bottom and enough toluene to form a $\frac{1}{4}$ inch layer on top. The flasks were then incubated at room temperature for periods varying in the different tests from two to four weeks. Suitable controls were also incubated. Examinations by the Breed method

were made at the beginning and from time to time during the periods of incubation to see if the spores would germinate.

The controls and the cultures were then examined chemically for hydrolysis of the casein by Sorensen's Formol Titration Method, after first precipitating the casein and removing ammonia by aeration. The details of the process will not be given at present since some modification will be necessary to get entirely satisfactory results. There were some indications of hydrolysis of the casein in 3 cases, in one of which the hydrolysis was marked. However, it is not certain that the hydrolysis was produced by caseinase, since the method as conducted gave some opportunity for chemical hydrolysis.

GELATINASE TESTS

Only four cultures were tested for gelatinase. Tubes of neutral, sterile, solidified 0.5 per cent phenol-gelatin were inoculated on the surface with 1 cc. of the spore suspension. Two to 3 cc. of toluene were placed on top of the suspension and the tubes incubated for seventeen days. Liquefaction of the gelatin was observed as follows:

	per cent
Culture 1	25
Culture 2	13
Culture 3	6
Culture 4	3

Smears were made of the suspensions and of the liquefied portions of the gelatin and examined carefully for spores and bacilli. No bacilli were observed but the spores were numerous. None of them showed evidences of germination. A test for amino acids by Sorensen's Formol Titration Method was also conducted as follows:

The gelatin was melted and the contents thoroughly mixed by agitation of the tube. Five cubic centimeters were added to some distilled water and the whole brought to neutrality to phenolphthalein. Ten cubic centimeters of neutralized formalin were then added and the acidity titrated with tenth normal potas-

sium hydroxide, using phenolphthalein as indicator. The following results were secured:

	cc. N/10 K.H
Control (uninoculated)	0.0
Culture 1	1.5
Culture 2	0.7
Culture 3	0.4
Culture 4	0.3

Both of these tests clearly prove that gelatinase was present even though germination did not take place.

From the foregoing experiments the tentative conclusion seems to be justified that bacterial spores do exhibit some enzymic activity even when there is no evidence of germination. If further experiments substantiate the above preliminary tests we may have to modify some of our ideas of spores. Aside from the theoretical interest of the question, it has some practical bearing on the preservation of foods and possibly also on the rôle of the spores in the soil.

UTILIZATION OF THE SALTS OF ORGANIC ACIDS BY THE COLON-AEROGENES GROUP

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The sanitary importance of the colon bacillus and its allies has led to numerous attempts to classify this complex group of bacteria, for the possible existence of certain types within the group which might be of especial sanitary significance has long been recognized. Among the various biochemical tests usually applied to the study of bacterial groups, the qualitative fermentation of carbohydrates and higher alcohols has for many years held the most prominent place and the list of the substances so employed has been an ever lengthening one as new products have been made available. The list of non-nitrogenous carbon-containing compounds which may be utilized as sources of energy need not be limited, however, to the commoner carbohydrates and higher alcohols since it is known that some microörganisms possess the ability to break down a great variety of carbon-containing substances of diverse structure.

The present investigation was undertaken as a study in bacterial nutrition to gain some idea of the utilization of the salts of the commoner organic acids by members of the colon-aerogenes group. Furthermore, should any separation of the various types within the group be effected, it was desired to determine whether this might be correlated with the distribution of these organisms in nature and therefore offer a possibility of practical use in the estimation of the sanitary quality of water.

There are many scattered references in the literature on the utilization of organic acids, or their salts, by various bacteria, and some attempts have been made to formulate systems of classifi-

cation based upon the fermentation of these substances. In spite of these suggestions, however, the organic acids have not found the general use to which they are seemingly entitled in the differentiation and classification of bacterial types. In 1892 Van Ermengem and Van Laer reported that the colon bacillus was able to break down formic, succinic, citric and tartaric acids with the formation of hydrogen, carbon dioxide and methane. Maassen (1895) endeavored to study the utilization of many of the commoner organic acids by adding them to a basic medium containing pepton and certain inorganic salts. The decomposition of the organic acids was indicated roughly by the development of an alkaline reaction and a greater turbidity than was shown by the same cultures in plain broth controls. Capaldi and Proskauer (1896) employed the ammonium salts of many organic acids in synthetic media which were used in a comparative study of *Bact. coli* and *Bact. typhosum*.

Much of the subsequent literature seems to have centered around the nutritive requirements and the products of metabolism of the colon bacillus and other members of the colon-typhoid group. Harden (1901), while studying the fermentation of carbohydrates and allied compounds by *Bact. coli-communis*, noted that formates were decomposed. Pakes and Jollyman (1901) studied the decomposition of sodium formate by various bacteria. Among the organisms capable of attacking formate were *Bact. coli-communis*, *Bact. lactis-aerogenes*, *Bact. cloacae* and *Bact. enteritidis*. It was estimated that 25 to 30 per cent of the formate was destroyed when 0.2 to 2 per cent was employed in the culture media. Grey (1913-1914) recognized that formic acid was an intermediate product in the decomposition of glucose by *Bact. coli* and that the formic acid itself was attacked in a secondary fermentation. Omelianski (1907) proposed the use of sodium formate broth to differentiate certain types. *Bact. coli*, a "Pseudodiphtheria bacillus" and the paratyphoids A and B attacked the formate with alkali production, while *Bact. typhosum* and *C. diphtheriae* lacked this property. Karczag and Móczár (1913, 1915) found that pyroracemic acid was attacked by *Bact. coli*, *Bact. paratyphosum* B, *Bact. enteritidis* and *Bact. typhi-murium*. Gas was

formed from the pyroracemic acid. Oxalacetic acid was attacked only very slightly by those organisms which possessed the property of decomposing pyroracemic acid (Karczag and Breuer, 1915). On the other hand, the above organisms, together with *Bact. pneumoniae*, produced a "hydrogen fermentation" from formic and glycolic acids (Karczag and Schiff, 1915). In the course of a study of several atypical *Bact. paratyphosum* B. cultures, Wagner (1913) reported that in plain nutrient broth plus 1 per cent pyroracemic acid an abundant gas formation was produced by typical *Bact. paratyphosum* B, *Bact. enteritidis* and *Bact. coli*. Altobelli (1914) reported that *Bact. coli* was capable of attacking the sodium salts of malic, tartaric and citric acids and converting them into carbonates. *Bact. typhosum* did not possess this property in the great majority of cases. Observations on the use of the above organic acid salts were extended to other bacteria.

More recently, several publications have served to emphasize again the utilization of organic acids. Braun and Cahn-Bronner (1921a) found that *Bact. paratyphosum* B was capable of growing readily in a chemically definite medium in which ammonium lactate formed the sole source of both nitrogen and carbon. Furthermore, the organism was carried through 100 successive passages in this medium in the course of one and a half years. The lactic acid could be replaced by succinic or citric acids but not by carbonates, formates or acetates. The same authors subsequently extended this work (1921b) to include *Bact. coli* and a number of other types. *Bact. coli* was able to develop through continuous passages in the lactate medium. Wagner (1920) employed a number of organic acids in an investigation of the biochemistry of the colon-typhoid group. The potassium or sodium salts of the acids were added to ordinary broth or agar and because of this it is difficult to interpret his results, although he evidently obtained undoubted utilization of certain of these organic acid salts by *Bact. coli* and *Bact. paratyphosum* A and B. Brown (1921), in connection with a study of the use of sodium citrate solution for collecting blood samples for cultural tests, made a series of observations on the growth of numerous species of bacteria, mostly pathogens, in plain broth containing 1 per cent sodium citrate. Upon compari-

son with plain broth controls it was found that some species grew more luxuriantly in the citrate medium while the growth of others was inhibited. Addition of a lead acetate solution to forty-eight hour cultures produced a voluminous white precipitate in the uninoculated control tubes and in those cultures exhibiting inhibition of growth. Where an increased luxuriance of the culture was evident there was little precipitate and in these cases Brown assumed that the citrate had been broken down and used by the organism. In the list of cultures which he tested in the citrate broth it is note worthy that *Bact. lactis-aerogenes* and *Bact. cloacae* showed the increased luxuriance of growth while the growth of *Bact coli* was inhibited. Taken in connection with the lead acetate test, we have here a suggestion that the aerogenes-cloacae types are capable of utilizing citrate while coli is not. Apparently the significance of this observation as applied to differentiation of the colon-aerogenes group and its *possible* application to sanitary water analysis were overlooked, for this promising opening was not followed.

Pesch (1921) made a study of selective media for the colon-aerogenes group and used several synthetic combinations in which either tartrate or citrate was supplied as the only source of carbon. *Bact. paratyphosum* B developed readily in either medium, *Bact. enteritidis* usually exhibited slight growth, 5 *Bact. coli* strains showed abundant growth in tartrate but refused to grow in the citrate medium, while *Bact. paratyphosum* A and *Bact. typhosum* refused to develop in any case. When he repeated the results in the citrate medium with 15 additional colon strains from urine and feces these also failed to grow.

In this country the work of Ayers and his associates has advanced considerably our knowledge of the utilization of organic acids. Ayers and Rupp (1918) called attention to the destruction of organic acids which may occur in a glucose medium simultaneously with the sugar fermentation. The reversion to an alkaline reaction exhibited by some cultures is due largely to the oxidation of the salts of the organic acids to bicarbonates and carbonates. Ayers, Rupp and Johnson (1919) used organic acid salts in a study of the classification of alkali-forming bacteria which do not attack the carbohydrates ordinarily employed in fermentation tests. As

a result of their work they suggest that since organic acids are suitable sources of carbon for many bacteria, they might be used as an extensive set of test substances in studies of the biochemical properties of microörganisms.

EXPERIMENTAL

From the foregoing citations it is evident that the organic acids offer a promising field for studies of the nutrition and metabolism of various groups of bacteria and at the same time may prove to be of considerable value in classification. Following this idea, it decided to determine the availability of a number of these acids as sources of carbon for members of the colon-aerogenes group. For this purpose a simple culture medium was adopted which would contain the organic acids as the only source of carbon. The basal medium employed throughout this work consisted of:

Distilled water	1000 cc.
NaCl	5.0 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram
$(NH_4)H_2PO_4$	1.0 gram
K_2HPO_4	1.0 gram

This combination gives a colorless clear solution having a pH of 6.7 to 6.8. To this solution the various organic acids were added and the reaction brought back to pH 6.8 by the addition of normal sodium hydroxide solution. Ammonium phosphate was used to supply the necessary nitrogen for development. An inorganic nitrogen-containing salt must be employed, since all carbon is to be excluded from the basal medium and to be derived solely from the test substances added.

Several preliminary tests showed that when a readily available source of carbon was added to the above combination of inorganic salts an abundant growth of both *Bact. coli* and *Bact. aerogenes* resulted. When the available carbon-containing compound was omitted, no growth was apparent. This is shown in table 1. Evidently in the foregoing combination of inorganic salts we have all the essentials for development of the colon-aerogenes group with the exception of an available carbon-containing compound. By adding the different organic acids, one at a time, to this solu-

tion their availability may be determined by noting the resultant development of the cultures.

The foregoing combination of inorganic salts cannot be regarded as an ideal one. It was merely found that when available nitrogen and carbon were present it permitted a moderately rapid and luxuriant growth of the colon-aerogenes cultures. In an effort to simplify the medium magnesium sulphate was omitted. This resulted in a poor growth of all the *Bact. coli* and *Bact. aerogenes* cultures. The addition of 0.01 per cent of calcium chloride, either with or without magnesium sulphate, was tried but since no greater luxuriance of growth could be discerned in either case as a result of its addition it was not employed regularly. These results should not

TABLE 1

Showing the effect upon growth of the addition of an available source of carbon to the solution of inorganic salts

COMPOSITION OF MEDIUM	ORGANISM USED FOR INOCULATION	RESULTANT GROWTH
Inorganic salts	*Bact. coli*	None
Inorganic salts	*Bact. aerogenes*	None
Inorganic salts + 0.2 per cent glucose	*Bact. coli*	Abundant
Inorganic salts + 0.2 per cent glucose	*Bact. aerogenes*	Abundant
Inorganic salts + 0.2 per cent lactose	*Bact. coli*	Abundant
Inorganic salts + 0.2 per cent lactose	*Bact. aerogenes*	Abundant

be held as indicating that calcium is not needed for development, since traces at least might have been present as impurities in the other salts.

Experiments with various organic acids

A number of different organic acids were available for the present study. These were added to the basic solution of inorganic salts in such amounts that when brought to a pH reading of 6.8 by addition of sodium hydroxide, the resulting concentration of the sodium salt would be approximately 0.2 per cent. The exact percentage concentration could not be calculated from the molecular weights as most of the organic acids here used are weak acids and their salts are partially hydrolyzed. Even though the pH value is at or near 6.8, it is not to be assumed that complete change of the

acid to its normal sodium salt has occurred. The actual amount of normal sodium salt present is somewhat less than that found by calculation but for the present purpose exact calculation based upon the dissociation constant is unnecessary.

Fifty representative cultures of the colon-aerogenes group, including strains from as many different sources as possible, were selected for the tests. The typical fecal *Bact. coli*, methyl red positive and Voges-Proskauer negative, was represented by 22 strains of either human or animal fecal origin. The *Bact. aerogenes* cultures, methyl red negative and Voges-Proskauer positive, were isolated from soil samples, water and grains, with the exception of a few fecal aerogenes cultures which were also included in the tests. A third set included 3 strains which upon repeated tests gave either a negative or a weakly positive Voges-Proskauer reaction. The methyl red test was consistently negative. The occasional weak Voges-Proskauer reaction, together with the general characteristics of the cultures, indicated a relationship with the aerogenes type. Another group consisted of 3 strains which were consistently methyl red negative—Voges-Proskauer negative. Aside from this the deportment of these cultures also showed a close similarity to the typical aerogenes type.

The different media were filled into test tubes, 8 to 10 cc. per tube and sterilized in the autoclave at 15 pounds pressure for fifteen minutes. All cultures were inoculated from young agar slants by transferring a small amount of growth on the tip of the straight wire. Incubation was at 30°C. The luxuriance of growth was recorded at definite intervals, usually one, two, and four and seven days after inoculation, although where negative results were secured this was frequently extended to thirty days.

A summary of the experiments with different organic acids supplied as the source of carbon is presented in table 2. The results are given here as recorded after seven days incubation since it is unnecessary to present the detailed observations made in each experiment. The several media used as controls are placed first. Here it is seen, as previously pointed out, that in the presence of an available source of carbon such as glucose or lactose,

TABLE 2

Development of the colon-aerogenes group with the organic acid salts as the only source of carbon

	BACT. COLI.		BACT. AEROGENES		RESEMBLING BACT. AEROGENES	
	Methyl red, + Voges-Proskauer, 0		Methyl red, 0 Voges-Proskauer, +			
	Fecal origin		Soil, water, grains	Fecal	MR, 0 VP, 0 or weak +	MR, 0 VP, 0
	Human	Animal				
NUMBER OF CULTURES IN EACH GROUP EMPLOYED IN TESTS	12	10	18	4	3	3
Control meda:						
Glucose, 0.2 per cent	+++ (10) ++ (2)	+++ (5) ++ (4) + (1)	+++ (18)	+++ (4)	+++ (3)	+++ (3)
Lactose, 0.2 per cent	+++ (5) ++ (5) + (2)	+++ (2) ++ (6) + (2)	+++ (18)	+++ (4)	+++ (2) ++ (1)	+++ (3)
No source of carbon	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.
Organic acid media:						
Sodium salt of, -Acetic	+++ (9) ++ (3)	+++ (3) ++ (7)	+++ (7) ++ (8) + (2) neg. (1)	+++ (3) ++ (1)	+++ (1) ++ (2)	+++ (3)

Propionic	++ (2) + (7) neg. (3)	++ (5) + (5)	all neg.	all neg.	+++ (1) neg. (2)	all neg.
n-Butyric	? or + (5) neg. (7)	+ (3) neg. (7)	all neg.	all neg.	+++ (1) neg. (2)	all neg.
n-Valeric	all neg.	all neg.	all neg.	all neg.	+++ (1) neg. (2)	all neg.
Iso-Valeric	all neg.	all neg.	all neg.	all neg.	+++ (1) neg. (2)	all neg.
n-Caproic	all neg.	all neg.	all neg.	all neg.	+++ (1) neg. (2)	all neg.
Succinic	+++ (12)	+++ (7) ++ (3)	+++ (15) ++ (2) + (1)	+++ (4)	+++ (3)	+++ (3)
Malic	+++ (11) ++ (1)	+++ (10)	+++ (16) ++ (2)	+++ (4)	+++ (3)	+++ (3)
Lactic	+++ (12)	+++ (8) ++ (2)	+++ (17) ++ (1)	+++ (4)	+++ (2) ++ (1)	+++ (3)

Increasing luxuriance of growth from slight to heavy turbidity is indicated by the plus signs +, ++, and +++ No evidence of growth is designated "neg." The numerals in brackets following these signs show the number of cultures exhibiting the particular luxuriance of growth indicated.

The observations given here are readings taken on the seventh day after inoculation. Incubation at 30°C.

TABLE 2—*Continued*

	BACT. COLI.		BACT. AEROGENES		RESEMBLING BACT. AEROGENES	
	Methyl red, + Voges-Proskauer, 0		Methyl red, 0 Voges-Proskauer, +			
	Fecal origin		Soil, water, grains	Fecal	MR, 0 VP, 0 or weak +	MR, 0 VP, 0
	Human	Animal				
NUMBER OF CULTURES IN EACH GROUP EMPLOYED IN TESTS	12	10	18	4	3	3
Glyceric	+++ (11) + (1)	+++ (9) ++ (1)	+++ (16) ++ (2)	+++ (3) ++ (1)	+++ (2) ++ (1)	+++ (2) ++ (1)
Citric	all neg.	all neg.	+++ (15) ++ (3)	+++ (4)	+++ (2) ++ (1)	+++ (3)
Tartaric	all neg.	all neg.	+++ (6) neg. (12)	+++ (1) neg. (3)	+++ (1) ++ (1) neg. (1)	+++ (2) neg. (1)
Mucic	+++ (8) ++ (1) neg. or ? (3)	+++ (8) + or ? (2)	+++ (13) ++ (5)	+++ (3) neg. (1)	+++ (2) ++ (1)	+++ (3)
Malonic	+ (8) ? (2) neg. (2)	+ (8) ? (1) neg. (1)	++ (9) + (8) ? (1)	++ (2) + (1) ? (1)	+++ (1) ++ (1) neg. (1)	++ (3)

Oxalic	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.
Benzoic	all neg.	all neg.	++ (2) + (3) neg. (13)	all neg.	+++ (1) + (1) neg. (1)	+ (1) neg. (2)	
Salicylic	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.	
o-phthalic	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.	

all the cultures develop and most of them attain a luxuriant growth. Without the available source of energy no development is apparent.

In the monobasic fatty acid series it was found that the lower acids supported growth while the higher acids were apparently not attacked. The results secured with acetic acid present a decided contrast to those given by valeric and caproic acids, while propionic and butyric acids seem to be intermediate in that they support a moderate or light growth of some of the coli type. All cultures, with one exception, developed in the acetic acid medium and in most cases attained a moderately heavy growth. A retarded development, due evidently to the acetate radical, was apparent. With the usual 0.2 per cent sodium acetate the first visible turbidity usually did not appear until thirty-six to forty-eight hours or more after inoculation and a heavy growth was not attained until the fourth or fifth day. An increase in the amount of sodium acetate from 0.2 to 0.4 per cent caused a correspondingly greater inhibition.

The results secured with propionic acid were especially interesting, for here many of the *Bact. coli* cultures produced a moderate growth while only one strain of *Bact. aerogenes* was able to develop. The coli strains developed very slowly and no turbidity could be discerned until the fourth or fifth day after inoculation. While this acid seemed to offer some promise of affording a means for the separation of the coli and aerogenes types, it was not investigated further at this time since some of the fecal cultures consistently refused to grow while one of the aerogenes type developed readily in every instance. Also, the slow development impaired considerably any usefulness which this acid might possess. It seems probable that the propionate radical exerts a retarding influence as did the acetate. In the presence of n-butyric acid a few of the *Bact. coli* cultures exhibited a meagre development. Whether this scanty growth was due to a limited availability of n-butyric acid or whether the butyrate radical exerted such a marked inhibitory effect as to prevent more than a slight growth was not determined.

Succinic, malic, lactic and glyceric acids were readily utilized by all cultures of both the coli and aerogenes types and usually supported an abundant growth.

Citric acid stands out as especially significant, for here there occurred a distinct separation in the behavior of the different types of the colon-aerogenes group. The *Bact. coli* cultures of fecal origin all refused to develop, while *Bact. aerogenes*, together with the several atypical strains, grew readily and the dense turbidity exhibited by these cultures was in striking contrast to the clear tubes which had been inoculated with the coli cultures. All aerogenes cultures multiplied readily, usually exhibiting turbidity within twenty-four hours and attaining a luxuriant growth by the third or fourth day. Several different samples of citric acid or its salts were employed with similar results.

Tartaric acid supported growth of some of the aerogenes cultures, though not all. *Bact. coli* showed no evidence of growth. Where a utilization of the tartrate was effected by *Bact. aerogenes*, the development was usually rapid and the cultures presented a dense turbidity. Mucic acid, which is quite similar to tartaric acid in structure, supported development of most of the colon strains and all but one of the aerogenes type. The remaining organic acids need little comment. In malonic acid many of the cultures produced a slight to moderate growth, the development of the aerogenes cultures usually being considerably heavier than that of the coli type. Benzoic acid supported the growth of a few of the aerogenes cultures, while oxalic, salicylic and orthophthalic acids were not utilized in any instance.

In all of the synthetic organic acid media, growth of both the coli and aerogenes types appeared first as a light uniform turbidity which gradually became denser and after several days partially settled to the bottom of the tube. Upon agitation a heavy uniform turbidity usually resulted, although a few of the cultures showed a tendency to clump and exhibited a more or less flaky appearance.

Determinations of the hydrogen-ion concentration were made at frequent intervals in all of the experiments. Utilization of the acids was accompanied by a decrease in the hydrogen-ion concen-

tration. In those cases where the organic acids were readily utilized and a luxuriant growth resulted, the pH of the culture was changed from the original 6.7 to 6.8 to a point usually about 8.4 to 8.8, within seven days. This was true of succinic, malic, citric, tartaric and mucic acids. Lactic acid always supported a heavy growth but the change to an alkaline reaction was never as pronounced, the final pH usually ranging from pH 7.2 to 7.6. Glyceric acid presented somewhat the same features, the aerogenes cultures attaining a pH of 7.0 to 7.4 and the coli cultures 7.4 to 8.4. In malonic acid *Bact. coli* developed only to the extent of a light turbidity and caused very little change in the reaction, while on the other hand some of the aerogenes cultures caused a decrease in the hydrogen-ion concentration to pH 8.2 to 8.8. It is possible that traces of impurities in the malonic acid may have been responsible for the slight growth of the colon strains. In every instance where development of the culture was not apparent the hydrogen-ion concentration remained unchanged. In young cultures a slight turbidity could be discerned before a change in the hydrogen-ion concentration could be detected by the colorimetric method.

A comparison of the structural formulae of the organic acids is of some interest.

Acetic	$CH_3 COOH$	Tartaric	$\begin{matrix} CHOH \cdot COOH \\ \vert \\ CHOH \cdot COOH \end{matrix}$
Propionic	$CH_3 \cdot CH_2 \cdot COOH$		
n-butyric	$CH_3 (CH_2)_2 \cdot COOH$	Mucic	$\begin{matrix} CHOH \cdot COOH \\ \vert \\ (CHOH)_2 \\ \vert \\ CHOH \cdot COOH \end{matrix}$
n-valeric	$CH_3 (CH_2)_3 \cdot COOH$		
iso-valeric	$(CH_3)_2 CH \cdot CH_2 \cdot COOH$		
n-caproic	$CH_3 (CH_2)_4 \cdot COOH$	Citric	$\begin{matrix} CH_2 \cdot COOH \\ \vert \\ C(OH) \cdot COOH \\ \vert \\ CH_2 \cdot COOH \end{matrix}$
Lactic	$CH_3 \cdot CH(OH) \cdot COOH$		
Glyceric	$CH_2 (OH) \cdot CH(OH) \cdot COOH$	Oxalic	$\begin{matrix} COOH \\ \vert \\ COOH \end{matrix}$
Succinic	$\begin{matrix} CH_2 \cdot COOH \\ \vert \\ CH_2 \cdot COOH \end{matrix}$	Malonic	$CH_2 \begin{matrix} COOH \\ \diagdown \\ \diagup \\ COOH \end{matrix}$

Malic	$CH(OH) \cdot COOH$ $CH_2 \cdot COOH$	Benzoic	$C_6H_5 \cdot COOH$
		Salicylic	$C_6H_4(OH) \cdot COOH$
		Ortho-phthalic	$C_6H_4(COOH)_2$ (1, 2)

Here it is seen that carbon is presented in a number of different combinations, in carboxyl, methyl and alcohol radicals and the benzene ring and with these radicals attached in different ways. The addition of the methyl groups to the carbon chain in the acetic acid series is accompanied by a corresponding decrease in growth. In a number of instances a slight change in the structure of the acid resulted in a decided difference in availability. Compare, for instance, propionic and lactic; oxalic, malonic and succinic; malic and tartaric or tartaric and mucic acids (table 2). On the other hand, several compounds of different structure have presented similar results. The explanation for the failure of *Bact. coli* to utilize citric acid does not seem apparent, since some of the radicals are similar to those contained in several of the acids which were readily utilized. It is possible that the addition of certain linkages to a compound may tie up or prevent the utilization of other carbon-containing radicals which would otherwise be available.

Experiments with citrates

Since it was found that the inability to utilize citric acid afforded a ready means for differentiation of the fecal *Bact. coli* strains from the other members of the colon-aerogenes group, further work was confined to a study of the usefulness of this acid. In addition to the usual citric acid neutralized with sodium hydroxide, several other salts of the acid were employed. Commercial preparations of potassium, ammonium and sodium citrate gave identical results. An experiment with 0.2 per cent sodium citrate (0.277 per cent sodium citrate $\cdot 5\frac{1}{2}H_2O$) showing the progressive development of the aerogenes type and the change in hydrogen-ion concentration is given in table 3. As brought out here, the distinction between the two types is usually apparent within twenty-four hours and becomes much more pronounced as the growth of the aerogenes cultures increases in luxuriance. In

TABLE 3

Showing the development of colon-aerogenes cultures in a synthetic medium containing 0.2 per cent sodium citrate as the only source of carbon

DESIGNATION OF CULTURES	LUXURIANCE OF GROWTH AFTER				pH OF MEDIUM AFTER			
	24 hours	48 hours	4 days	7 days	24 hours	48 hours	4 days	7 days
Bact. coli. (fecal)								
Human 1	0	0	0	0	6.7	6.7	6.7	6.7
Human 2	0	0	0	0	6.8		6.8	6.8
Human 10	0	0	0	0	6.8	6.7	6.8	
Human 11	0	0	0	0	6.8			6.8
Human 12	0	0	0	0	6.8	6.7	6.8	6.8
Human 13	0	0	0	0				6.8
Human 14	0	0	0	0	6.7		6.8	
Human 15	0	0	0	0				6.8
Human 16	0	0	0	0			6.8	
Human 17	0	0	0	0	6.8		6.8	
Human 18	0	0	0	0		6.8	6.8	6.8
Human 19	0	0	0	0	6.8	6.8	6.7	6.8
Rat 1	0	0	0	0	6.7			6.8
Rat 2	0	0	0	0	6.8			6.9
Rat 3	0	0	0	0	6.8			6.8
Guinea-pig 1	0	0	0	0	6.8			6.8
Guinea-pig 3	0	0	0	0	6.8			6.8
Guinea-pig 4a	0	0	0	0	6.8			6.8
Rabbit 1	0	0	0	0	6.8			6.8
Rabbit 5	0	0	0	0	6.7			6.8
Rabbit 8	0	0	0	0	6.8			6.8
Sheep 2	0	0	0	0	6.8			6.8
Bact. aerogenes (soil, water and grains)								
P_1	+	++	++	++	6.8	7.0	8.4	8.6
P_3	++	+++	+++	+++	7.0	7.6	8.8	9.0
2050B	++	++	+++	+++	6.9	7.2	8.2	8.6
RCP	++	+++	+++	+++	7.1	7.4	9.0	9.0
L	++	+++	+++	+++	7.2	7.4	8.0	8.8
2203	++	++	+++	+++	7.0	7.4	8.0	8.4
RC-5	+	+++	+++	+++	7.2	7.5	8.6	9.0
Va.1B	++	+++	+++	+++	7.4	8.2	8.8	9.0
Va.1C	+	++	++	+++	6.8	7.2	7.6	8.4
PGF1	+	++	++	++	7.0	7.2	7.6	8.4
PGF3	+	+++	+++	+++	6.9	7.1	8.0	8.6
PGF4	+	+++	+++	+++	6.9	7.4	8.0	8.6
PGF6	++	+++	+++	+++	7.2	7.6	8.6	9.0
12755	+	++	+++	+++	7.0	7.6	8.8	9.0

TABLE 3—*Continued*

DESIGNATION OF CULTURES	LUXURIANCE OF GROWTH AFTER				pH OF MEDIUM AFTER			
	24 hours	48 hours	4 days	7 days	24 hours	48 hours	4 days	7 days
Bact. aerogenes (soil, water and grains)—*Continued*								
79482	++	+++	+++	+++	7.0		8.6	9.0
690	+	++	+++	+++	6.8	7.0	7.8	8.4
Rice 1	+	+++	+++	+++	7.0	7.4	9.0	9.0
Flax 1	+	+++	+++	+++	6.9	7.2	8.6	9.0
Fecal aerogenes								
Rabbit 2a	+	++	+++	+++	6.9	7.1	7.4	8.4
Rabbit 6	++	+++	+++	+++	6.9	7.4	8.6	9.0
Rabbit 9	+	++	+++	+++	6.8	7.0	7.8	8.4
Guinea-pig 8B	++	+++	+++	+++	7.2	7.6	8.4	9.0
Voges-Proskauer reaction variable, methyl red negative								
PGF 5	+	++	++	++	7.0	7.5		8.8
2050 A	+++	+++	+++	+++	7.4		8.6	9.0
gp.5	0	++	++	+++	6.7	7.0	7.7	8.4

Increasing luxuriance of growth is represented by the plus signs, +, ++ and +++. 0 signifies no visible growth.

this and preceding experiments the temperature of incubation was 30°C. Similar results have been secured at 37°C. With the cultures used in the present investigation either temperature may be employed, although 30°C. is to be recommended for general use as an occasional aerogenes culture may grow with difficulty at body temperature.

Optimum concentration of citrate

In all of the previous work 0.2 per cent citrate was adopted arbitrarily. Since it is well known that varying concentrations of a salt may cause either an inhibition or stimulation of growth it is necessary to obtain definite knowledge of the effect produced by different amounts of citrate upon the behavior of *Bact. aerogenes* and *Bact. coli*. For this purpose the combination of inorganic salts described in the first part of the paper was employed and to this sodium citrate was added in varying concentrations.

Controls which contained no citrate were also employed in every instance. Where the larger amounts—1 and 3 per cent—of citrate were used the hydrogen-ion concentration of the medium was slightly lowered and it was necessary to adjust the reaction to the usual pH 6.7 to 6.8 by means of HCl. For the purpose of

TABLE 4

The effect of various quantities of sodium citrate upon the rate of growth of Bact. aerogenes

CULTURE DESIGNATION	TIME REQUIRED TO SHOW VISIBLE GROWTH IN SODIUM CITRATE CONCENTRATIONS OF							PLAIN BROTH CONTROL
	3 per cent	1 per cent	0.5 per cent	0.1 per cent	0.01 per cent	0.001 per cent	None (control)	
	pH-6.8	pH-6.8	pH-6.8	pH-6.8	pH-6.7	pH-6.7	pH-6.6	pH-6.7
	hours	*hours*	*hours*	*hours*	*hours*			*hours*
Bact. aerogenes:								
Rabbit 2a	48	19	14	13	12	neg.*	neg.	5
Rabbit 9	24	12	9	9	9	neg.	neg.	6
G. P. 8b	24	10	10	9	9½	neg.	neg.	6
P 1	22	18	14	13	11	neg.	neg.	6
RCP	16	9½	10	9½	10	neg.	neg.	5
RC 5	24	12	10½	11	12	neg.	neg.	5
Va 1B	22	10	10	8	11	neg.	neg.	4½
PGF6	20	16	11	10	11	neg.	neg.	6
79482	22	12	11½	10	11	neg.	neg.	6
690	18	11	11	9½	9	neg.	neg.	5
Flax 1	22	10	9½	9	10	neg.	neg.	7
Average time for aerogenes	23.8	12.68	10.95	10.09	10.5			5.59
Bact. coli (controls):								
Human 1	neg.*	neg.	neg.	neg.	neg.	neg.	neg.	neg.
Human 10	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
Human 18	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

* Tubes remaining clear at expiration of 4 days were recorded as negative.

determining the comparative rapidity of development in citrate media of various concentrations the procedure of Holm and Sherman (1921) was followed. This consists simply in using the time required by the organisms for production of visible turbidity as a measure of the rate of growth. To insure a uniform inoculation, the inorganic salt-citrate media were tubed in 10 cc. quan-

tities and all tubes in a series representing the range of citrate tested were inoculated with one small loopful, 1 mm. loop, of a twenty-four-hour culture grown in either 0.1 citrate or 0.1 per cent glucose synthetic medium. Eleven strains of *Bact. aerogenes* and three of *Bact. coli* were used. All cultures were incubated at 30°C. and observations made at frequent intervals to note the first appearance of visible growth. The results of this experiment are given in table 4.

It is evident that the higher concentrations of citrate markedly retard the growth of *Bact. aerogenes*. This is especially noticeable in the 3 per cent concentration where the average time required for the aerogenes cultures to produce a visible turbidity was 23.8 hours. The inhibitive effect of citrate has been noted by Holm and Sherman (1921) who found that 0.2 molar sodium citrate in a one per cent pepton solution retarded the growth of *Bact. coli*. The experiment given in Table 4 shows that the inhibitive effect is decreased as the amount of sodium citrate is reduced until at a concentration of 0.1 per cent the most rapid growth occurs. In the 0.01 per cent citrate medium a slightly longer average time is required to show visible growth, though this difference is almost negligible. The 0.001 per cent citrate and the control tubes without citrate failed to show visible growth. Evidently the 0.001 per cent sodium citrate is insufficient to support the continued multiplication necessary to produce a visible cloudiness. To compare the development in plain broth with that in the citrate medium a series of tubes of beef extract pepton broth was included. Here the growth was much more rapid than that in any of the inorganic salt-citrate media.

While the above experiment shows that the highest rate of growth takes place in the lower concentrations of citrate (0.1 and 0.01 per cent) since the least time is required to show visible turbidity, it does not give an idea of the luxuriance of growth attained in the various amounts of citrate after the first visible turbidity has appeared. Thus, although growth was slow in appearing in the 1 and 3 per cent concentrations, ultimately a much more luxuriant development was attained here than in the smaller amounts of citrate. The cultures in 0.01 per cent never showed

more than a light growth while in the 0.1 per cent citrate medium the turbidity was not quite as dense as that exhibited by the 0.5 per cent concentration. Evidently, then, the smaller amounts of citrate are insufficient to support the aerogenes cultures to the limit of their ability to develop. Therefore, to secure the best results in separating the coli and aerogenes types we must choose

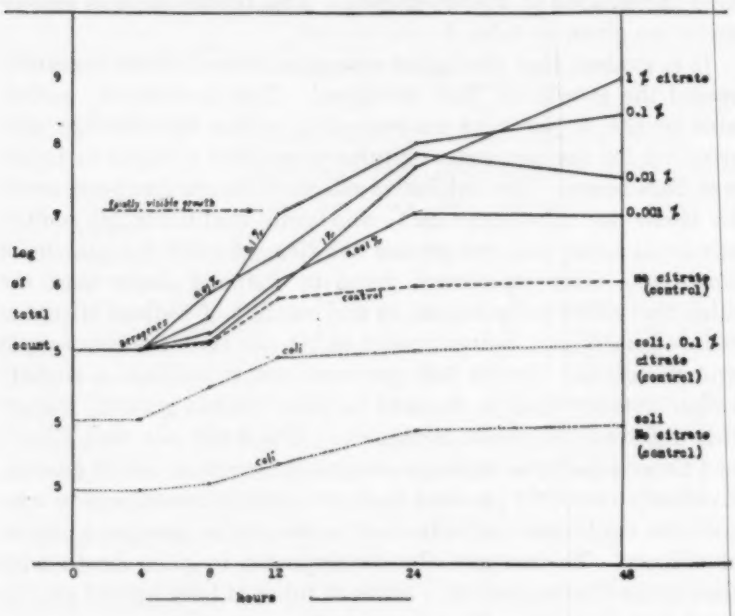

CHART 1

Bact. aerogenes cultures in citrate media of various concentrations are indicated by solid lines (————), without citrate by dash line (– – – –). The *Bact. coli* cultures used as controls are indicated by dotted lines (.....).

a concentration of sodium citrate small enough to permit a fairly rapid development of *Bact. aerogenes* and at the same time one which will supply sufficient available carbon to allow the organism to attain a luxuriant growth.

To show this more clearly additional experiments were performed in which plate counts were made at different time inter-

vals. The medium employed was the same as that previously used and was put up in flasks containing 50 cc. each to which the various amounts of sodium citrate were added. Several sets of flasks, each representing a series of varying citrate concentrations, were inoculated with different cultures of *Bact. aerogenes* and *Bact. coli*. The results of a representative experiment are shown in chart 1. Here we have a confirmation of the previous experiments, for within the first twelve hours after inoculation the rate of growth is most rapid in the 0.1 and 0.01 per cent concentrations of sodium citrate and these are the first to exhibit visible growth. It is not until after twenty-four hours that the lower concentrations are surpassed by the 1 per cent citrate which steadily increased in numbers while the others had practically reached the peak of development or had decreased. The total counts obtained at the end of forty-eight hours presented a series of gradation comparable to the density of growth as shown by macroscopic observation.

The slight multiplication of *Bact. aerogenes* in the control flask which contained no citrate and also that of *Bact. coli* in the 0.1 per cent citrate, as well as without the citrate, was somewhat surprising. It is believed that the increase in numbers in the controls may be explained by the presence of impurities in the inorganic salts. The increase in numbers of *Bact. coli* in the 0.1 per cent citrate cannot be accounted for so readily since it is slightly greater than that in the controls and may be explained either by an incomplete utilization of the citrate molecule, insufficient to produce a visible growth, or else, and more probably, by impurities in the sodium citrate as well as in the inorganic salts.

From the foregoing experiments on the influence of various amounts of sodium citrate in an inorganic salt medium upon the rate and luxuriance of growth of *Bact. aerogenes*, it is evident that a concentration of between 0.1 and 0.5 per cent will allow a fairly rapid development and at the same time will supply sufficient carbon to permit the attainment of an abundant growth. In most of the subsequent work 0.2 per cent sodium citrate (0.277 per cent sodium citrate $\cdot 5\frac{1}{2}H_2O$) has been employed.

Effect of small inoculations into citrate medium

It is well recognized that many of the more "fastidious" microorganisms will grow on certain media only when considerable amounts of old growth have been carried over into the new cultures and that when only a few cells are transferred no growth results. In the previous experiments inoculation was usually performed by transferring on a straight platinum wire a small amount of young culture from agar slants. Occasionally this procedure was varied by using the loop to transfer from one liquid citrate medium to another. Since such methods result in carrying over several hundred thousand or several million living cells with also small amounts of nitrogenous matter representing dead cells and perhaps traces of enzymes, it was deemed advisable to subject *Bact. aerogenes* to a more rigorous test of its ability to initiate growth. To accomplish this purpose the amount of inoculum was reduced so that each tube of citrate medium received only a few cells. These small numbers were secured by transferring a small amount of growth from a twenty-four-hour agar slant through several 10 cc. dilution tubes of sterile water. From the final dilution tube one loopful was inoculated into 10 cc. of citrate medium and another loopful was introduced into a agar tube which was immediately plated. By this procedure it was found that the total number of living cells introduced into the 10 cc. tubes of citrate medium varied from 82 to 670, i.e., the numbers in the tubes immediately after inoculation varied from 8 to 67 per cubic centimeter. All cultures were placed at 30°C. and examined from time to time to note the first appearance of visible growth and also the luxuriance of subsequent development.

Twenty strains of *Bact. aerogenes*, mostly of soil and water origin, were tested in this manner and in every instance they all developed readily and eventually produced a dense turbidity. Owing to the extremely small inoculum, the appearance of the first visible growth was delayed. Turbidity never appeared before eighteen hours and usually not before twenty-four to thirty hours, while a few cultures required thirty-six to forty-eight

hours to produce a visible growth. After the appearance of the first visible cloudiness the turbidity rapidly increased and the cultures became as luxuriant as those which had been inoculated in the usual manner. This would seem to indicate clearly that *Bact. aerogenes* finds in the citrate radical a readily available source of carbon and that it experiences no difficulty in utilizing it for its own needs. Also, it serves to check the results secured by the usual method of inoculation.

Permanence of the ability to utilize citrate

Bacteriological literature is replete with accounts of variability evidenced by different organisms. A few workers have even gone so far as to claim that the various biochemical tests, such as fermentation of sugars, etc., are valueless for distinguishing microbic types. Although the bulk of opinion is opposed to this view, it is necessary nevertheless to study the constancy of a particular biochemical property when advocating its use for the separation of types. The question may be raised whether *Bact. aerogenes* cultures ever lose their ability to utilize citrate or whether the typical fecal *Bact. coli* may acquire this power. Several experiments were undertaken to answer these points in so far as possible.

In the first place, to determine the ability of *Bact. aerogenes* to sustain continued multiplication in the citrate medium, 10 representative cultures were selected and each was transferred through 50 successive transplants in a medium consisting of the usual inorganic salts and 0.2 per cent sodium citrate. Transfers were made at twenty-four-hour, or occasionally at forty-eight-hour intervals. Throughout the entire series of transplants no decrease in either the rapidity or luxuriance of the growth was apparent. Secondly, to determine whether colon-aerogenes cultures would exhibit the same behavior in a citrate medium upon several isolated tests at different intervals, all the available stock cultures were tested on several different occasions during the course of a year. The stock cultures were kept on agar slants and transferred directly to the usual medium of inorganic salts

plus 0.2 per cent citrate. Altogether 41 cultures of fecal origin (methyl red positive, Voges Proskauer negative), 26 from soil, water and grains (methyl red negative, Voges Proskauer positive) and 6 atypical strains were subjected to from 3 to 5 different tests. In every case the behavior toward citrate remained constant and no change either toward a loss or gain of this power could be detected. Indeed, several details were noted which tended to show remarkable constancy. In the collection of cultures there were 2 strains which exhibited a delayed fermentation of citrate. Visible turbidity was not apparent usually until the third or fourth day after inoculation, although the cultures attained a luxuriant growth after that. These 2 cultures, in the several tests in various citrate media made at different times during the course of a year, gave on each occasion the same delayed fermentation. From the foregoing experiments it is seen that *Bact. aerogenes* was capable of utilizing citrate as a source of carbon throughout a continued period of multiplication and that no gain or loss of the power to attack citrate could be found in a series of 73 colon-aerogenes group cultures.

DISCUSSION

From the foregoing experiments it is seen that many of the organic acids offer an available source of carbon for the colon-aerogenes group and in the presence of suitable nitrogen and certain inorganic salts these acids are broken down with resultant development of the organisms. Evidently the organic acids are utilized both as a source of energy and as a source of carbon for construction of the bacterial cell.

The differentiation of the coli and aerogenes types on the basis of citric acid utilization is of special interest. It is well known that these types may be separated on the basis of glucose metabolism (Rogers and associates, 1914 and 1915; Clark and Lubs, 1915; Levine, 1916). Also, the writer (Koser, 1918) has previously shown that a differentiation may be made on the basis of ability to derive nitrogen from uric acid. The present investigation shows that, in addition to the foregoing methods, these types

can be separated by their ability to utilize certain salts of citric acid. *Bact. aerogenes* and several aerogenes-like strains were able to derive carbon from sodium, potassium and ammonium citrate, while the coli type of fecal origin did not possess this power. The luxuriant growth exhibited by the aerogenes cultures in a citrate medium was in direct contrast to the negative results secured with *Bact. coli*. It is noteworthy that *Bact. aerogenes*, which is considered on the basis of certain cultural properties and extensive sugar fermentations to be the most primitive type of the group (Winslow, Kligler and Rothberg, 1919), is able to derive nitrogen and carbon from certain sources which are not available for *Bact. coli*. This seems to constitute further evidence of the primitiveness of the aerogenes type, if we may assume as Winslow and his coworkers have done, that the course of evolution has been marked by progressive loss of fermentative power.

In regard to the question of the value of citrate differentiation in practical water analysis, the writer believes that this must be left open for the present, since there is not a uniformity of opinion in regard to the value of the commonly used methyl red and Voges-Proskauer tests in determining the sanitary quality of water. To determine the usefulness of a synthetic citrate medium for separation of fecal and non-fecal types of the colon group in routine water analysis several additional lines of work must be completed. A larger series of cultures than that employed in the present investigation should be collected from fecal and non-fecal sources and their behavior in the citrate medium studied. In addition, the relative incidence of the citrate utilizing and non-utilizing types in waters of different sanitary quality must be determined.

Aside from any possible applications in sanitary water analysis, the present investigation shows that the organic acids are entitled to a wider use by bacteriologists than that accorded them in the past. Many of the recent studies in bacterial nutrition have emphasized the utilization of nitrogenous bodies, such as amino acids, purines, ammonium salts, etc., and considerable information concerning the fundamental nitrogenous require-

ments of various groups of microörganisms has been brought out. It is apparent that studies of the utilization of carbon-containing compounds, such as the organic acids, should also prove exceedingly valuable in arriving at an accurate knowledge of the nutritive needs of bacteria and that the organic acids offer a valuable series of test substances for the differentiation and classification of bacterial groups.

SUMMARY

The utilization of various organic acids by the colon-aerogenes group was studied. The organic acids were employed as sodium salts and constituted the only sources of carbon in a simple medium composed of inorganic salts including a suitable source of nitrogen. The utilization of the salts of the various organic acids was shown by development of the cultures and by the production of an alkaline reaction.

A number of the salts, such as those of acetic, succinic, malic, lactic, mucic and glyceric acids supported an abundant growth of the colon-aerogenes cultures. Others, as n-valeric, iso-valeric, n-caproic, oxalic, salicylic and ortho-phthalic gave negative results. Several organic acids supported the growth of some but not all of the colon-aerogenes group. Propionic acid permitted a slight or moderate growth of many of the fecal *Bact. coli* strains while the *Bact. aerogenes* cultures, with one exception, were negative. Tartaric acid was readily utilized by a few of the *Bact. aerogenes* strains while the remainder of this type, as well as *Bact. coli*, gave no evidence of growth. Benzoic acid supported a light growth of a few of the aerogenes type.

The most striking results were obtained with citric acid. Here the fecal *Bact. coli* type consistently failed to develop while the *Bact. aerogenes* cultures multiplied readily and produced a luxuriant growth. The hydrogen-ion concentration of the aerogenes cultures decreased from an initial pH 6.7 to 6.8 to 8.4 to 9.0 while that of the coli cultures was unchanged.

Further work with the salts of citric acid showed that identical results could be secured with either potassium, ammonium or sodium citrate.

The citrate, while supplying available carbon for the aerogenes cultures, exerts a retarding effect upon growth. This becomes more pronounced as the amount of citrate is increased. A concentration of 0.1 to 0.5 per cent sodium citrate was found to be most useful for securing growth by *Bact. aerogenes*. Smaller amounts do not supply sufficient available carbon to permit an abundant growth while larger amounts retard the development.

A small inoculation resulting in the introduction of 8 to 67 living cells per cubic centimeter of the citrate medium was sufficient for *Bact. aerogenes* to initiate development.

Ten *Bact. aerogenes* strains were transferred through fifty consecutive cultures in an inorganic-salt citrate medium. No diminution of growth was apparent. Further experiments showed that the ability to utilize citrate is not readily gained or lost and is evidently a stable character.

The organic acids constitute a set of test substances which should be very useful in the differentiation and classification of various groups of bacteria.

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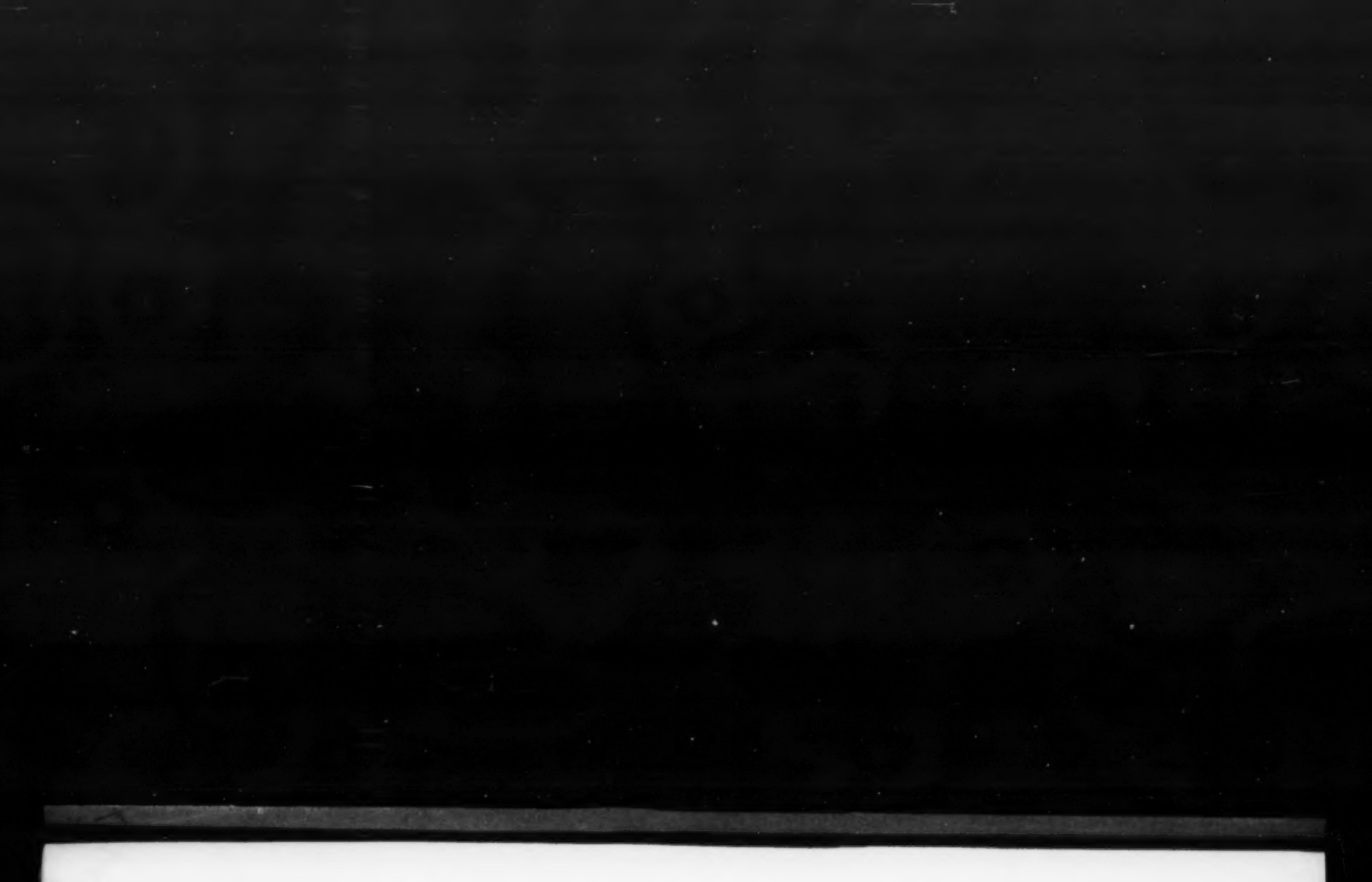
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